

# STUDIES ON RIGOR MORTIS

## PART I. OBSERVATIONS ON THE MICROSCOPIC AND SUBMICROSCOPIC STRUCTURE <sup>1</sup>

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NINETEEN FIGURES

Since muscle is one of the most plastic tissues in the body its histological image is more affected by previous treatment and history than that of any other. It is well known that the type of fixation and method of preparing sections influence the image considerably and that extensive change takes place in the muscles after the death of the individual. Such changes are collectively referred to as rigor mortis.

It has been recently reported by Bogoroch ('49) that the striations of skeletal muscle disappear 5 hours after death, but reappear and are prominent at 24 hours after death. These results were based on the study of stained sections in ordinary light. In the studies of Gerendas and Matoltsy ('48a, b, c) post mortem histological modifications are contrasted with the effect of extracting muscles with various solutions. They report that putrefaction results in a fiber that is homogeneous and shows positive birefringence which condition they attribute to the decomposition of an "N-protein" in the originally isotropic band. Striations are not apparent in their illustration but no definitive statement is made in the text.

Present studies were undertaken to follow step by step the changes taking place in muscle after the death of the

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animal. The amount of shortening was studied with the aid of kymograph records while the histological structure was studied by means of stained and unstained sections observed in ordinary and polarized light. This paper is concerned only with the histology of muscle as observed at various intervals after death.

#### MATERIALS AND METHODS

Fifty adult rats were used from which were taken 100 paired gastrocnemius muscles. The animals were sacrificed by exsanguination via the femoral arteries under light ether anesthesia. The muscles were carefully dissected free, with the origins remaining attached to the severed distal end of the femur. The muscles were then mounted in moist chambers for the purpose of recording the length changes, to be described in a subsequent paper (Smith, '50). Muscles were tetanized, maintained at room temperature, and at 37°C.

These treatments considerably affected the type and degree of shortening but had no appreciable effect on the histological structure. After various intervals post mortem the muscles were fixed in Bouin's or 10% formalin and paraffin sections were made in the usual manner. The sections were stained with iron hematoxylin or left unstained, and all were mounted in balsam.

#### OBSERVATIONS

For convenience of description the muscles are arranged in 4 groups. Group I is composed of muscles fixed from 2-10 hours post mortem, group II 10-20 hours, group III 20-30 hours, and group IV 30-50 hours.

##### *Group I — Fixed 2-10 hours post mortem*

The fibers of the muscles in this group were in excellent histological condition and in general might be characterized as normal. In polarized light the characteristic alternating anisotropic and isotropic bands demonstrate the fundamental periodic nature of striated muscle (fig. 1). When this bire-



fringence is observed in terms of its effect on the first order red the anisotropic areas raise the color to blue while the isotropic areas have no effect and hence are red of the first order. The birefringence of the A bands is therefore positive in sign.

In view of the findings of Bogoroch ('49) that the striations disappear after 5 hours, a special search was made for fibers with faint or absent striations. Before 10 hours post mortem only one or two isolated fibers were found in which the presence of striations was doubtful. All other fibers except those about to be described were normal, with striations evident in all. The peculiar fibers comprised only an extremely low percentage (less than 0.1%) of the fibers present, and this frequency of unusual fibers is about what occurs in sections of any muscle. These unusual fibers first come to attention because of their apparent increased birefringence. In figure 2 are seen several such fibers as they appeared under polarized light and at first glance one might think that they were without striations, however careful adjustment of the focus revealed that the fiber is crossed by a series of extremely fine markings. If the same fibers are viewed under ordinary light (fig. 3) there can be no doubt of the presence of a periodic cross-striation. When compared with the fiber at the left side of figure 5, these striations appear to be finer in structure and placed closer together. This fiber is in a contracted state. In figure 4 is illustrated a fiber found at 6 hours post mortem in which the striae are not immediately evident but with careful adjustment of the focus it was possible to resolve the markings throughout the fiber. Another example is taken from a muscle at 9 hours post mortem and in figure 5 the field is illustrated as it appeared with a high dry objective. At this magnification the larger fiber appears to be without markings in polarized light, however when this same fiber is viewed with oil immersion and polarized light (fig. 6) it is seen to present the usual periodicity. Furthermore, when viewed with ordinary light (fig. 7) the fiber again quite clearly presents periodicity. This is also a very much

contracted fiber. In the muscles examined up to 10 hours post mortem there were only a few isolated parts of fibers in which the usual periodic structure could not at all be discerned. These areas were located in contraction nodes. In figure 8 is shown such a contraction node as viewed under polarized light, showing the characteristic widening of the fiber at the location of the node. On either side of the node the fiber shows clearly cross-striations but in the center of the node, although it is strongly birefringent no further details of structure could be resolved.

The fibers of the muscles in this group reacted to the Heidenhain's iron hematoxylin in the normal manner. There were the usual variations in the stainability of entire fibers or parts of fibers but in all cases striations were apparent. Most of the stained muscle fibers in this group appeared to be in an early stage of contraction and many showed the characteristic separation of the A band into two parts with the intervening non-staining H line (figs. 10 and 11). Neither the Z nor M lines appeared to take the stain. From these stained sections the fact that the fibers were in varying states of contraction was clearly apparent, but such states should be considered as normal. The findings of Bogoroch ('49) that the striations disappeared could not be substantiated either with stained sections viewed in ordinary light or with unstained sections viewed in polarized light.

#### *Group II — Fixed 10-20 hours post mortem*

During this period certain changes are to be seen in the sections which are indicative of post mortem degeneration. The more superficial fibers do not exhibit either birefringence or striations (fig. 9). These changes extend to a depth of 15-20 fibers. Since the deeper fibers of the muscle show a different pattern of the loss of birefringence one might suspect that the two effects are caused by different mechanisms. It is possible that this loss of birefringence and striations in the superficial part of the muscle is due to the action of bac-



teria which were seen here in large numbers. In addition to the changes in the birefringence these peripheral fibers also show a strong affinity for the stain, giving rise to very dark fibers, without apparent structure.

In the interior of the muscle there are also degenerative changes but these have the form of only localized isotropic areas within the fibers (fig. 12). The fiber directly under the cross hairs in figure 12 runs continuously from the top to the bottom of the print and its interrupted appearance is due to the isotropic areas appearing black. It should be noted that in the unaffected areas the fiber retains a strong birefringence and periodicity. Many central areas also show a change in staining reaction, by taking the stain much less and giving rise to a fiber (picture) that may be characterized as "soft" (fig. 13).

In this group of muscles, fibers with complete loss of striations occur in the superficial part of the muscle only, while in the deeper part the loss of structure was observed only in restricted portions of individual fibers.

### *Group III — 20-30 hours*

In general the changes noted for group II are evident here, but have become more extensive. The area of the superficial degeneration is much greater and the isotropic patches in the deeper fibers are more extensive. The necrotic areas are still rather small (10-20 sarcomeres) and do not necessarily extend entirely across the fiber indicating that this necrosis is probably not related to the structure of the muscle fiber. In this group are to be seen some fibers that show evidence of longitudinal disorganization and reduced birefringence. When they are examined in various planes of focus the reduced birefringence appears to be the result of slipping of the fibrils resulting in their misalignment. The reduced birefringence is probably the result of anisotropic and isotropic areas being superimposed and somewhat neutralizing each other (fig. 14).

The superficial part of the muscle which has since very early been presenting a general lack of optical activity, now shows discrete, optically very active areas. They will be referred to as "anisotropic granules" (fig. 15). These "anisotropic granules" appear first in areas that are otherwise completely isotropic. They usually occur in multiples showing a definite periodicity and are the first degenerative phenomena to bear any relationship to the normal structural pattern of the fiber. There are several species of these granules. A few do not show extinction, while some have positive and others negative birefringence.

In this group of muscles the degenerative processes have proceeded to the point of producing misalignment within the fiber and granules with great optical activity.

#### *Group IV — 30-50 hours*

As would be expected, in these muscles the degenerative processes have proceeded farther, and affect a larger percentage of the fibers than in the previous groups. The whole muscle is now involved and there is no sharp difference between the superficial and the deep parts either in optical activity or staining. There are still some areas in which the fibers are essentially normal, but these are definitely in the minority. The areas showing birefringent granules have increased in number and size. In figure 16 will be seen the common condition as exemplified by this fiber in which double refraction is absent except in the areas occupied by the highly birefringent granules. These granules show either positive or negative birefringence and have the elements of a pattern reminiscent of the sarcomeres. It is possible that such granular fibers represent early stages of the condition to be next described. However, since it was impossible to locate these granules accurately in any of the standard bands of the sarcomere, the true interpretation must await further investigation.

The extreme condition encountered in these studies shows fibers with a definite periodic organization, although with



considerable granulation (figs. 17, 18 and 19). Many of these fibers present both positive and negative birefringence in an orderly sequence where the A band still maintains a weak positive birefringence, while the adjacent I band now presents strong negative birefringence.

The identity of the A and I bands can be established by the examination of a fiber in which the changes in optical activity have occurred on one side only, while the other side maintains the original condition. Such a condition is shown in figures 18 and 19 where the upper part of the fiber shows the weak A band which has positive birefringence and adjacent to it are the isotropic I bands. As these bands are followed to the lower edge of the fiber it is observed that the A band remains unchanged and retains its weak positive birefringence. As the I band is followed into the lower edge of the fiber it is seen suddenly to develop a strong birefringence which when tested with the selenite plate proves to be negative in sign. Here the identification of the bands is unquestionable and it is demonstrated that the negative birefringence is located in the I bands. These highly birefringent areas can be further resolved by enlargement when it can be seen that they are related to the fibrillar structure and that they have the form of elongate granules which is characteristic of the I band. As the proteins and carbohydrates are the more labile of the constituents of muscle and would be expected to decompose first, the idea comes to mind that this very decided change in the optical activity of the I band may be due to the persistence of a lipoid. To my knowledge the presence of a lipase in muscle has not been reported. It may be that ordinarily the negative birefringence of this material is concealed by the presence of a material with positive birefringence and that here the former material is made evident by the decomposition of the latter.

In order to distinguish the effects of bacterial action from those of autolysis several representative sections were stained by Gram's method. Such a procedure brings about a visualization of the bacteria and demonstrated that they are

mainly confined to the outside of the muscle. Up to 12 hours post mortem only a few isolated individual bacteria were seen in the deeper parts of the muscle while at the periphery there was excellent growth. After 40 hours post mortem there was luxuriant growth of bacteria at the periphery penetrating to a depth of 5-10 fibers in but in the deeper parts of the muscle they were found only as a few here and there. It is not believed that these deeper organisms contributed much to the changes in structure of the muscle as small groups (10-20) were seen adjacent to fibers which maintained their structure well and in places where the fibers were completely broken down the organisms were often completely absent. These observations imply that the histological changes seen in the periphery of the muscle may be the result of bacterial action, but the changes in the deeper parts of the muscle are probably the result of autolysis.

#### DISCUSSION

In no case were the striations completely absent during the 6-12 hour period as described by Bogoroch ('49) although a special search was made in an effort to observe such a condition. Since the work of Bogoroch ('49) was done with stained preparations viewed in ordinary light, the reported results are perhaps indicative of a change in the affinity of the sarcomeres for the stain and hence reflect a chemical change within the sarcomere rather than an anatomical change. It should be noted that the period (5-24 hours) during which the effects described by her were most apparent, coincides well with the period during which maximal shortening is taking place (Smith, '50, accompanying paper) and the pH is changing rapidly (Bate-Smith, '39). It is therefore possible that the effect so described is simply that of a lack of affinity for the stain during the shortening phase. The sarcomere is a morphological element, and even though there is still some doubt as to which of the subdivisions are caused by interfacial differences and which are caused by the presence or absence of material, it is nevertheless a definite,



permanent although protean structure. It is here demonstrated that the sarcomere persists until putrefaction has reached a late stage, and even then vestiges will be seen for some time. Modifications in the appearance of the sarcomeres due to functional changes have been described (Jordan, '20; Speidel, '37), but only rarely do these changes embody more than the local reversible reduction in the visibility of the striations.

The changes in birefringence are not unexpected except in the later stages of disorganization. In the central part of the muscle, apparently not subject to bacterial action, the birefringence remains fairly strong for a long time. When localized breakdown occurs the isotropic nature of such areas is as would be expected from the ultrastructure of muscle. It has been demonstrated (Fischer, '44) that muscle is a highly organized system of birefringent micellae, and destruction of either the micellae per se or the aligning bonds would result in an area which appears to be isotropic. Such isotropicity does not necessarily infer that the micellae have been destroyed, because it could also result from their random orientation. The areas of negative birefringence which are to be seen in the terminal stages of this study are somewhat confusing. Their appearance here does not correspond to the results as described by Matoltsy and Gerendas ('48c), as these authors reported that a putrefying muscle fiber is a homogeneous positively birefringent structure without striations. After treatment with Weber's solution with the addition of KI they then found that the positive birefringence of the I bands (as well as A bands) was reduced and there then appeared a negative I band. Such muscles then showed negative I bands alternating with positive A bands. In the present studies of fibers that had undergone putrefaction none of them showed a homogeneous positive birefringence, and all of them maintained evidence of striae until liquefaction set in. Negative birefringence of two types was observed in these studies. Isolated islands of negatively birefringent material were observed in muscles where the putre-

factive processes had obliterated all evidence of the normal structure. As these could not be related to the structure of the sarcomere they probably represent fragments of decomposed sarcomere constituents. The second type of negative birefringence was seen as large areas of fibers which showed patches of positive and negative birefringence. In many areas the two appeared simultaneously and it was observed in such places that the A band showed very weak positive birefringence while the adjacent I band showed powerful negative birefringence. This condition has been reported by Matoltzky and Gerendas ('48c) after extraction with Weber's and KI solution. The experiments were based on the supposition that the I band is isotropic due to the simultaneous occurrence of both positively and negatively birefringent materials and that when such a system is extracted with Weber's solution, to remove the positively birefringent material, the negative birefringence of the I bands would then become apparent. Putrefaction as observed here did not in any case produce a homogeneous fiber with positive birefringence, but did produce a fiber in which the I bands were negatively birefringent while the A bands showed a weakened positive birefringence.

To interpret such a finding one must consider the nature of the ultrastructure in the sarcomere. The electron microscopic observations of Hall, Jakus and Schmitt ('46) demonstrate that the fibril is composed of extremely fine filaments. It is important to note that these filaments are not confined to just one sarcomere but extend through several sarcomeres. Since all parts of a sarcomere are made up, at least in part, of fine longitudinal filaments, the subdivisions (A, I, and Z) may be due to the presence of some material in addition to these filaments. The filaments presumably consist of birefringent material. The problem of the segmentation of the sarcomere therefore becomes that of explaining why it is not uniformly birefringent, and in determining what factor or factors are responsible for the reduction of birefringence in certain areas. The continuity of some birefringent ma-



terial was implied in the observation of Schmidt ('37) that the I band is only relatively isotropic, and the additional observations by the same author, Frey-Wyssling ('48), and the writer, that the area of the Z line frequently exhibits marked birefringence. It is unlikely that restriction of the filaments to the A band would give rise to such a phenomenon. The one-half I bands adjacent to the Z lines are then the areas of reduced birefringence and one must seek here for a cause of this relative isotropicity. Such a phenomenon could be the result of the positive filaments having superimposed on or between them at the I band a material which is negatively birefringent, or a positively birefringent material oriented at right angles to the fiber axis. The result of either situation would be a subtraction of retardations giving rise to an apparently isotropic area. Compensation with a selenite plate indicates that there is present in the negative areas described here a material in which the extraordinary ray is vibrating at right angles to the muscle fiber. Whether such a direction of vibration is due to a substance which is negative in sign or whether it is due to a substance oriented perpendicular to the long axis of the fiber was not determined. From the fact that negative birefringence is not generally seen unless the muscle is mounted in balsam, one may deduce that the form birefringence is probably positive in sign ( $n$  balsam 1.55,  $n$  water 1.33). Such speculations lead to the contention that the material observed here as negatively birefringent is possibly composed of micellae oriented parallel to the long axis of the fiber, but which have a negative crystalline birefringence. This negative crystalline birefringence is probably very weak as it is seen only after considerable autolysis of the fiber has greatly reduced the positive birefringence. But even then it is clearly evident only when the form birefringence of the filaments is further reduced by mounting in a medium with an index circa 1.53. It is well known from the work of Fischer ('44) that the turning point of the imbibition hyperbola is located at this index. From the regularity of his curves it might be

assumed that the turning point of any birefringence of a negative nature must be similar to that of the positively birefringent material, but regardless of the turning point, when mounted in  $n$  1.55 the negative birefringence is strong enough to overcome the weakened positive birefringence of the longitudinal fibers.

The concept of a negatively birefringent substance located in the I band has previously received support from other workers. Dempsey et al. ('46) postulate the presence of some lipoidal material located in the I band, which is soluble in hot alcohol. Although these authors did not demonstrate the occurrence of a substance with negative birefringence, its original presence and later removal was inferred from a "marked increase in the birefringence of the isotropic segment" after alcohol extraction. The presence of a material of lipoidal nature in the I bands was further indicated by the positive results of the Smith-Dietrich reaction. Additional evidence that lipoid material is present in the I band has recently been secured by Richter ('50). He finds that the isolated sarcomere is made up of two chemically distinct layers, a cortical osmophilic isotropic layer beneath which in a smaller globule is located a discrete body of osmophobic anisotropic material. The osmophilic material is presumably lipoid in nature, but isotropic. When such sarcomeres are arranged serially as they are in a muscle fiber there would result an alternation of optical properties due to the presence or absence of the anisotropic material.

#### SUMMARY

1. In these balsam mounted paraffin sections of mammalian muscles undergoing post mortem changes the striations do not disappear and later reappear. The striations are evident and relatively normal until putrefaction has resulted in complete destruction of the tissue.

2. The birefringence of the muscles gradually diminishes, the peripheral fibers losing this property early, while in the more centrally located fibers it persists for a longer time.



3. Areas of negatively birefringent material appear showing some relationship to the periodicity. In the extreme condition fibers with negatively birefringent I bands alternating with weakly positive birefringent A bands are to be seen.

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## PLATE 1

### EXPLANATION OF FIGURES

1 An unstained section of muscle 4 hours post mortem in polarized light. The bright bands are the A bands, the dark ones the I bands. In this and all of the subsequent figures (except fig. 17) the cross hairs indicate the position of the Nicol's prisms.  $\times 970$ .

2 An unstained section of muscle 2.2 hours post mortem in polarized light. Fine striations are apparent above and below the cross hairs.  $\times 430$ .

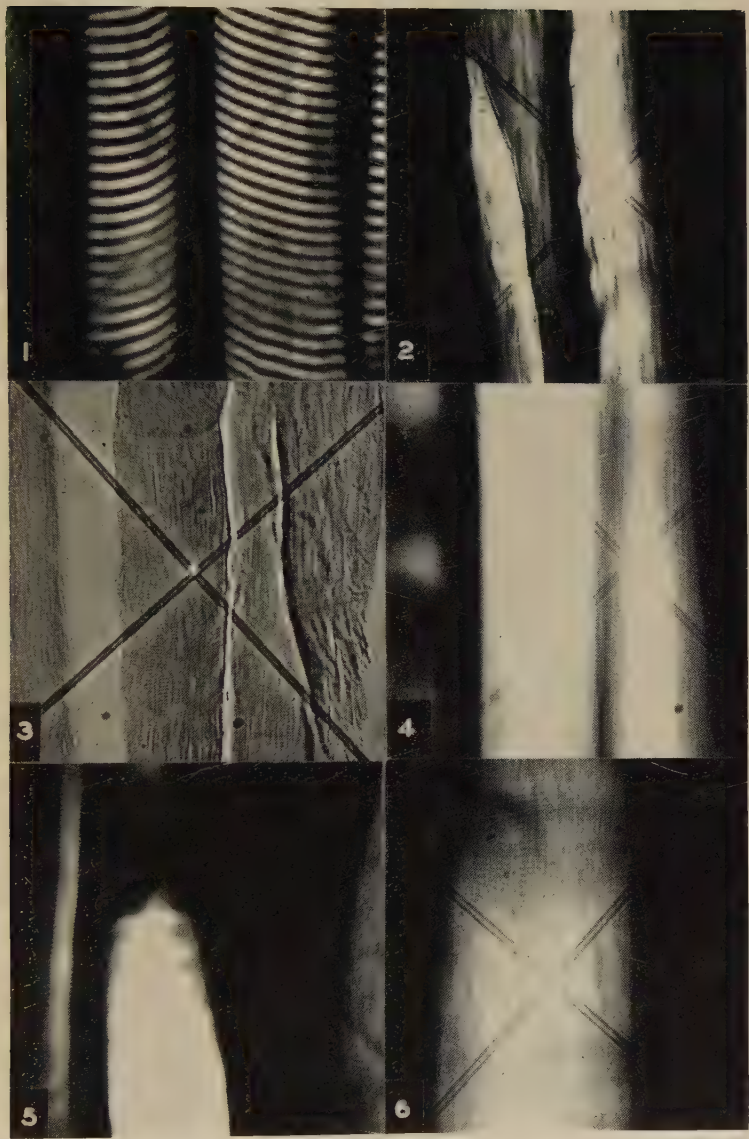
3 The same area as in figure 2, but here viewed in ordinary light.  $\times 430$ .

4 An unstained section of muscle, 6 hours post mortem in polarized light. Striations are faint but exist throughout the fiber.  $\times 970$ .

5 An unstained section of muscle, 9 hours post mortem in polarized light. Striations not apparent in the larger fiber at this magnification.  $\times 430$ .

6 Greater magnification of the larger fiber of figure 5. Periodicity is now evident in polarized light.  $\times 970$ .





## PLATE 2

### EXPLANATION OF FIGURES

7 The same fiber as in figure 6, in ordinary light. Striations are unquestionable.  $\times 970$ .

8 An unstained section of muscle, 9 hours post mortem in polarized light, showing a contraction node. In the brightest portion of it, striations could not be resolved.  $\times 970$ .

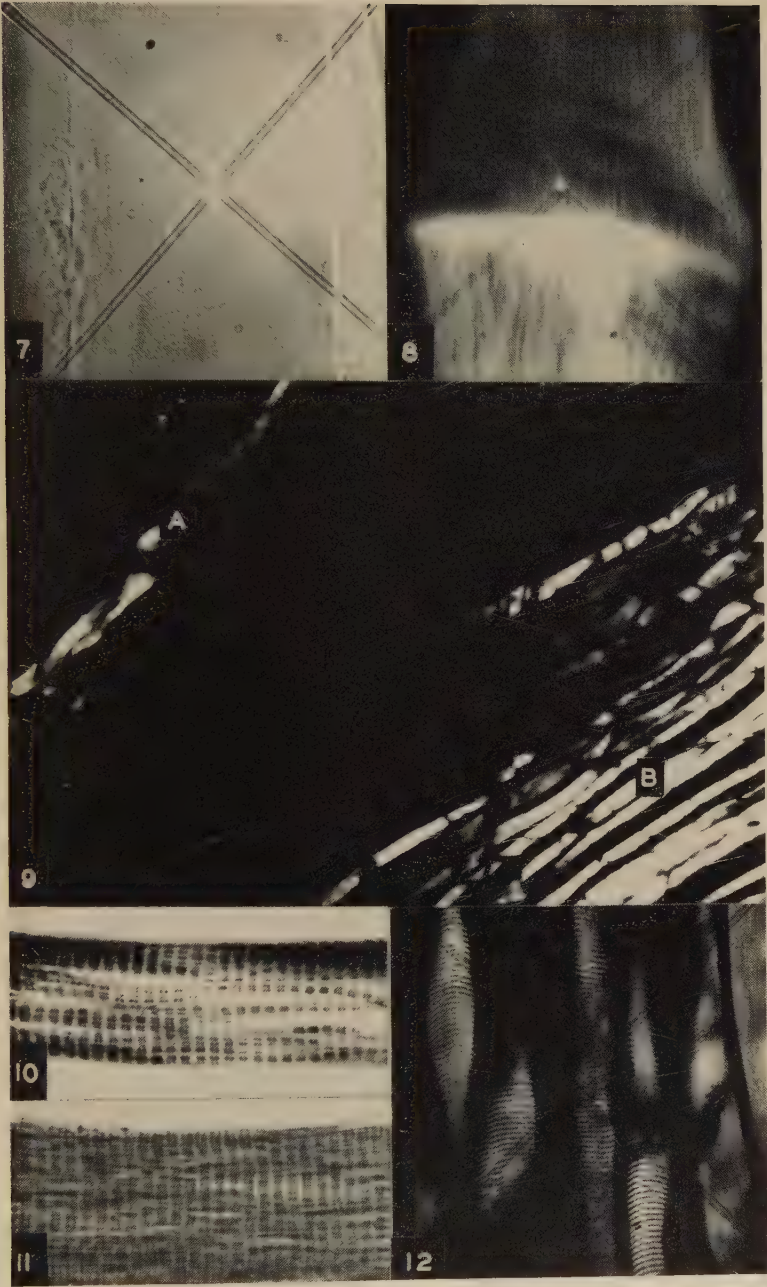
9 An unstained section of muscle, 11 hours post mortem in polarized light. A marks the position of the superficial tendon of origin, which is consequently the outer edge of the section. B marks a deeper portion of the muscle. The area between A and B is occupied by muscle fibers, but because most of them have lost their birefringence this region appears to be empty in polarized light. From B to the center of the section the fibers continued to show birefringence.  $\times 100$ .

10 A section of muscle, 4.3 hours post mortem, stained with Heidenhain's iron hematoxylin, in ordinary light. This fiber is in an early stage of contraction as evidenced by the splitting of the A band into two parts thereby revealing the H band.  $\times 1125$ .

11 A section of muscle, 4.3 hours post mortem, stained with Heidenhain's iron hematoxylin, in ordinary light. These sarcomeres are also in an early stage of contraction, but this muscle takes the stain less vigorously than was the case with the previous.  $\times 1125$ .

12 An unstained section of muscle 11.5 hours post mortem in polarized light. The interrupted appearance of the fiber directly under the cross hairs is due to the fact that many parts of this fiber have completely lost their birefringence and consequently are not to be seen in polarized light.  $\times 430$ .





### PLATE 3

#### EXPLANATION OF FIGURES

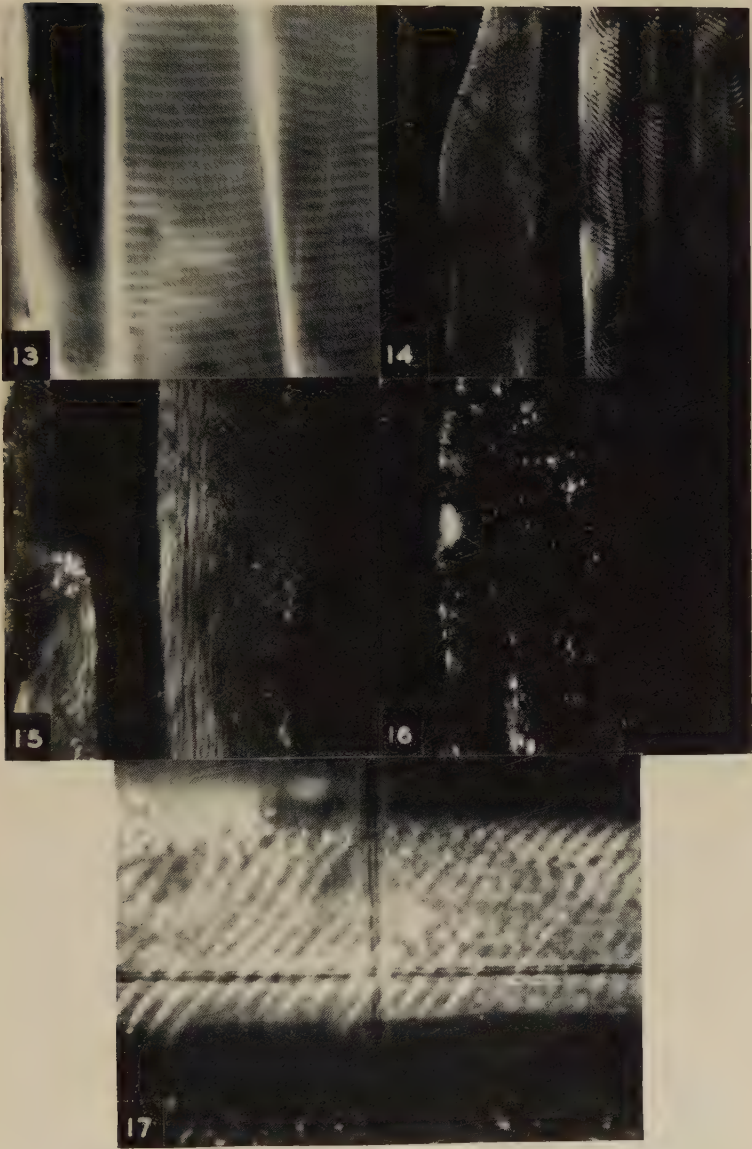
13 A section of muscle 11.5 hours post mortem, stained with Heidenhain's iron hematoxylin in ordinary light. The lack of contrast between the A and I bands is apparent, and such a condition at lower magnification might be mistaken for lack of striae.  $\times 1125$ .

14 An unstained section of muscle, 25 hours post mortem in polarized light. The fiber to the left of center is an illustration of the loss of birefringence due to misalignment of the fibrils.  $\times 430$ .

15 An unstained section of muscle, 25 hours post mortem in polarized light. A fiber showing the highly birefringent granules which are occurring in otherwise isotropic areas.  $\times 430$ .

16 An unstained section of muscle, 38 hours post mortem in polarized light. Further development of the highly birefringent granules. They are more numerous, larger, and stronger.  $\times 430$ .

17 An unstained section of muscle 42 hours post mortem in polarized light. The extremely bright bands are negatively birefringent I bands. The cross hairs are placed at  $45^\circ$  to the Nicols.  $\times 1455$ .



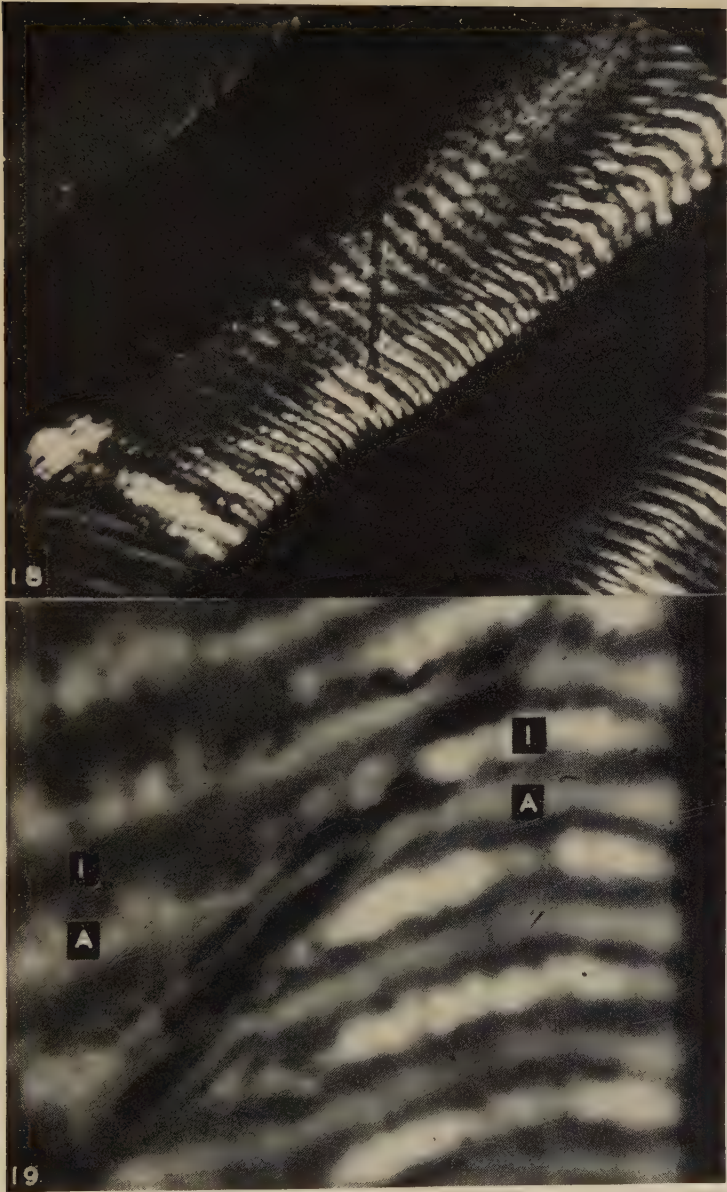


## PLATE 4

### EXPLANATION OF FIGURES

18 An unstained section of muscle, 43 hours post mortem in polarized light. The upper left half of this fiber shows the normal pattern of bright A bands alternating with dark I bands. The lower margin of the fiber shows the development of negatively birefringent I bands. The identification of the normal bands can be made in the upper half of the fiber.

19 An unstained section of muscle, 43 hours post mortem in polarized light. An enlarged portion of figure 18 demonstrating the identification of the bands. On the left side of this figure the normal (by this time only slightly), birefringent A band is seen, bounded on either side by the dark I band. As these bands are followed to the right of the figure the A band is seen to remain unchanged, while the I band develops a strong birefringence which is negative in sign.  $\times 3400$ .







## STUDIES ON RIGOR MORTIS

### PART II. QUALITATIVE OBSERVATIONS ON THE POST MORTEM SHORTENING OF MUSCLES

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TWO FIGURES

The changes occurring in muscles after death have been of general interest for some time but there is little agreement as to the sequence of events within the fiber during the development of rigor mortis. Most authors indicate that the muscles stiffen, but the relationship of this to post mortem shortening is not entirely clear. Since it is well known that when a living muscle contracts it not only shortens but also becomes hard, it becomes difficult to separate the hardness of contraction from that of rigor. The stiffness which develops in a muscle post mortem might then be a stiffening without shortening, or a stiffening associated with shortening. Wells ('20) attributes these phenomena to both causes, viz.: "All forms of muscle, striped, smooth, and cardiac, undergo this change (rigor mortis), which is shown by a shortening and thickening of the muscle, which also becomes opaque and hard." Green ('05) also believes both phenomena to be concurrent and states that the muscles "become firm and somewhat shortened as though in a state of permanent contraction."

Hoet and Marks ('26) clearly state their opinion, "In ordinary rigor mortis the muscles undergo a progressive shortening and hardening, the shortening being particularly well seen when the antagonistic muscles are cut before the rigor

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is complete." More recently, Shennan ('35) states: "The muscles contract during the development of rigidity." Moore ('45) states simply that "within two to three hours after death the muscles become stiff," and a similar opinion is expressed by Anderson ('48), Smith ('42), and Delafield and Prudden ('09). E. C. Bate-Smith has studied changes in the pH and hardness ('30a), modulus of elasticity ('39), and heat production ('30b) as muscle undergoes rigor, but he does not regard change in length of the muscle as a normal concomitant of rigor (Smith and Brendall, '47). However it is indicated in the text that isolated muscles have been observed to shorten with the development of tension during rigor and that these events can be correlated with the disappearance of ATP. Szent-Gyorgyi ('47) reports that the breakdown of ATP will cause precipitation of actomyosin and hence bring about a weak contraction. He defines rigor mortis as a slightly contracted rigid condition of actomyosin.

It is felt that a more definite opinion is needed as to whether there is generally a shortening of the muscles after the death of the animal. The present paper is concerned with the qualitative determination of length changes and the extent to which these reactions may be modified by temperature or tetanization of the muscle. Histological studies of the structural changes occurring post mortem in these muscles have been reported (Smith, '50).

#### MATERIALS AND METHODS

The paired gastrocnemius muscles of 45 adult rats were used in this study. The animals were sacrificed by exsanguination via the femoral arteries after light ether anesthesia. The skin of the thigh and leg was removed, the tendo calcaneus severed, and the muscle dissected proximally to its origin from the femur. The knee joint was disarticulated, the femur severed through the distal third and all other muscles were removed to free the bone and the attached gastrocnemius muscle. The muscles were then mounted individually in Har-

ward muscle warmers which were lined with blotting paper moistened with Ringer-Locke solution. The femurs were tied to the supports and the tendon attached via a thread to a heart lever. The lever was slightly overbalanced and recorded on a smoked drum at a  $10\times$  magnification. A long paper kymograph moving at 4 mm/hour was used to record the changes in length.

The muscles were observed in three groups: (1) at room temperature ( $25^{\circ}\text{C}.$ ), (2) at room temperature after tetanization, and (3) at  $37^{\circ}\text{C}.$  The muscle from the opposite leg of each animal was used as the control. In the tetanized group the muscle from the right leg was removed and the muscle of the left leg was tetanized in situ after tenotomy. The electrical tetanization was carried out via the nerve until the muscle no longer responded. After such treatment this muscle was removed and it and the control were then mounted in separate moist chambers for recording the length changes as described above. The effect of the environmental temperature on the development of rigor was studied by mounting the muscles from the right and left legs in separate moist chambers. One chamber was placed in a water bath at  $37^{\circ}\text{C}.$  while the other remained at room temperature. A few muscles were studied by the same technique at  $0^{\circ}\text{C}.$

#### OBSERVATIONS

The post mortem length changes were followed in 89 muscles and all of them showed a definite shortening followed by a variable pattern of relaxation. In about 50% of the muscles the shortening was evident in the first hour while the remainder showed a period of slight lengthening before the onset of shortening. Aside from this latter phenomenon all of the muscles behaved in a comparable manner. The maximum shortening was attained in 8 hours at room temperature and in 4 hours at  $37^{\circ}\text{C}.$  The maximum was maintained briefly and within two hours there was evidence of lengthening. The lengthening proceeded fairly rapidly for



a few hours, after which in about half of the cases the rate of lengthening became extremely slow.

The characteristic sequence of events exhibited by a muscle followed at room temperature are to be seen in figure 1. The curve begins with a period of elongation of almost three hours during which the writing lever drops below the initial level. This is followed by a definite shortening of about 4 hours duration, after which the record levels off somewhat.

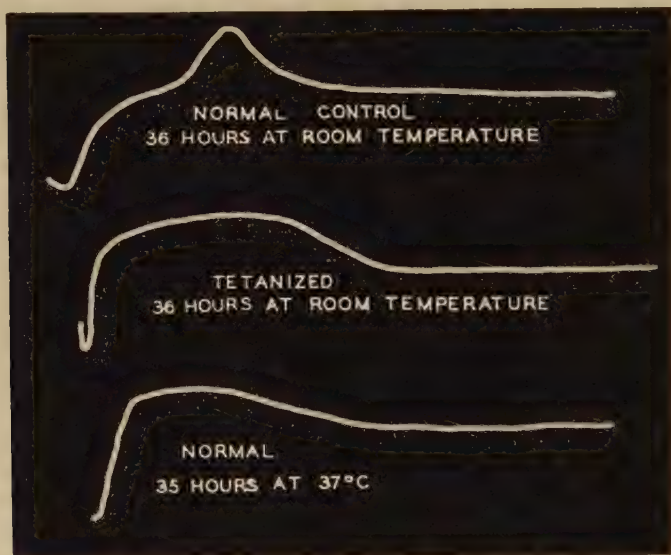


Fig. 1 Photograph of tracings of kymograph records showing the patterns of shortening presented by the rat gastrocnemius muscles during the development of rigor mortis at room temperature and at  $37^{\circ}\text{C}$ ., as well as after tetanization.

This is followed by a second period of relatively rapid shortening of about two and one-half hours duration. The maximal shortening is maintained for one hour and then lengthening begins. There is a rather sharp drop in the record, indicating that the process is initially fairly rapid, later the muscle lengthens only slowly.

The curve produced by averaging the records of all of the nontetanized muscles which were run at room temperature

demonstrates the consistency of these general features (fig. 2) in spite of the fact that the reactions of the muscles were asynchronous. The initial relaxation is evident and the compound nature of the ascent is apparent if the slope at one to three hours is compared with the slope at 3-6 hours. The plateau is not apparent but the low increments during the 7-9 hours indicates its location. The initial phase of the

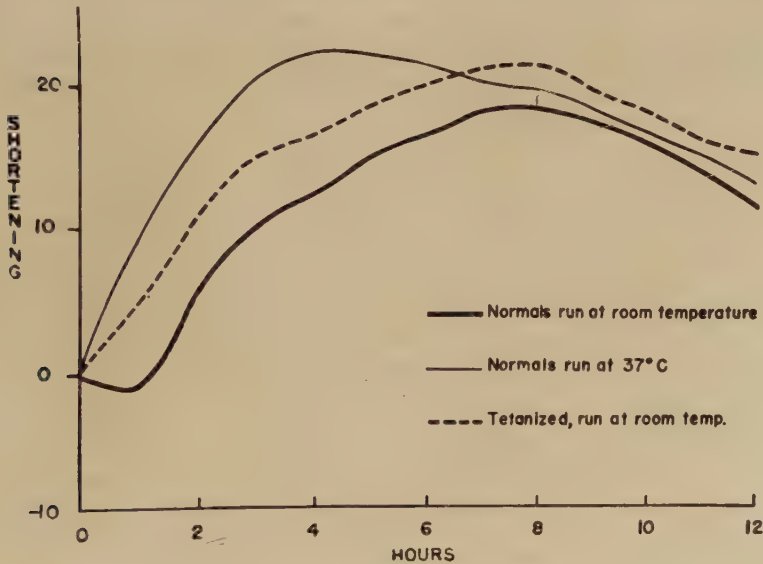


Fig. 2 Each curve represents the average of all the muscles recorded under each condition.

decline is fairly rapid and failure to reach the original level is apparent.

When the muscles were kept in an environment of  $37^{\circ}\text{C}$ . during the development of rigor the accelerating effect of temperature is indicated (fig. 1). The early post mortem changes within the muscle are speeded up to such an extent that the initial elongation is no longer observed. The initial slope is much steeper leading to an earlier attainment of the maxima but the lengthening process seems to be little af-

fect. The compound nature of the shortening process is not apparent and the only trace of it is the rather elongated plateau. The curve produced by averaging the records of all of the muscles which were run at 37°C. (fig. 2) differs from that produced at room temperature in several respects. The slope of the curve is immediately positive and steep with no indication of the compound nature. The maximum shortening which is reached in 4 hours, is slightly greater than that achieved by either the room temperature controls or the tetanized muscles. The slope of the post maximal decline is however not appreciably different from that of either of the other curves and is still well above the initial level at 12 hours.

Tetanzing the muscles via the nerves before mounting modified the record surprisingly little. The initial period of elongation was usually obliterated or of very brief duration (fig. 1) and consequently the initial shortening occurred earlier than in the controls. The average initial shortening is not appreciably different, showing both a slope and dual nature similar to that of the controls (fig. 2). The average maximal value is somewhat higher than the room temperature controls, and occurs after approximately the same post mortem interval. The slope of the post-maximal lengthening period is essentially the same as shown by the other muscles.

#### DISCUSSION

The work of E. C. Bate-Smith ('30a, b, '39, '47) has established certain correlations between intrinsic chemical and physical processes and the development of rigor mortis. In his studies either the development of hardness or the increase in modulus of elasticity was used as the criterion for the development of rigor. He has shown ('47) that the increase in the modulus of elasticity is related to the breakdown of ATP and that as this substance is depleted, rigor develops. Evidence was produced which indicated that a lowering of the pH to circa 6.1 will augment the destruction of ATP, but



the pH change is not a *sine qua non* as "alkaline" rigor can also occur. In view of this evidence supporting the Szent-Gyorgyi theory that a minimum level of ATP is essential in order that a muscle may not be contracted, it seems strange that such a system would be independent of environmental temperature. Furthermore, it is peculiar that tetanization, which is presumed to lower the level of ATP within the muscle, did not affect the rate of development of rigor as determined by the changes in modulus of elasticity.

When shortening of the muscle is used as a criterion of the development of rigor, some of these apparent inconsistencies can be harmonized. A biochemical basis for considering shortening to be associated with rigor rests on the Szent-Gyorgyi studies of actomyosin, in which he found that the absence of ATP causes a precipitation of actomyosin accompanied by a shortening. The work of Bate-Smith ('47) confirming that of Erdos (cited by Szent-Gyorgyi, '47) makes it clear that rigor is certainly associated with a reduction in the amount of ATP. Since lack of ATP causes a precipitation of actomyosin leading to hardness and lowered elasticity, one wonders why it is not accompanied by a shortening in the muscle. Although Bate-Smith depreciates post mortem shortening, he has not published records demonstrating that it does not take place.

In the present studies, post mortem shortening of a muscle is considered to be a normal accompaniment of rigor mortis, since all of the muscles tested showed a definite and clear-cut shortening. That this shortening is associated with the same group of phenomena as discussed by Bate-Smith is evidenced by the similarity between the average curve of shortening obtained from muscles going into rigor at room temperature and his published curves of hardness, pH changes, modulus of elasticity, and heat production. These latter phenomena all show a similar type of curve characterized by very little change before two hours post mortem, steep slopes of rapid change between 2 and 8 hours, and a leveling off of the curves between 8 and 12 hours. The pattern

is almost identical to that produced by the post mortem shortening of the muscle. A similar correlation can also be established between the rate of destruction of ATP and the beginning of shortening. Most of the ATP is destroyed in two to three hours (150–200 minutes) (Smith, '47) and while the shortening usually does not begin before one hour, about half of it has occurred in three hours, and the remainder after three hours. Apparently the level of ATP is reduced sufficiently at the end of one hour to initiate the shortening of rigor, and as the level falls the rate and amount of shortening increases. It can also be seen that at about 8 hours post mortem all of these phenomena are reaching their maxima almost simultaneously (in spite of the fact that pH and heat were observed at 21°C. and the others at 25°C.). This correlation of length change data with those of Bate-Smith's studies leads the author to conclude that the sclerometer values, modulus of elasticity and shortening are each a measure of rigor mortis.

It is believed that measurement of shortening may be a method which reflects with greater accuracy the chemical activities within the muscle undergoing rigor. From figures 1 and 2 it is evident that there are differences in the patterns of shortening between the normal muscles and those tetanized or run at a higher temperature. The averages of such records show that these differences are consistent enough to be evident here in spite of the fact that the individual records are asynchronous. If the processes evidently involved in rigor mortis are the result of the breakdown of ATP, it follows that treatment which will reduce the amount of ATP or speed up its destruction should accelerate the development of rigor. Bate-Smith reports that he was not able to correlate changes in the moduli of elasticity with the environmental temperature at which the muscle is undergoing rigor. In the present studies it is shown that the normal muscles which were allowed to go into rigor at 37°C. shortened at a much higher rate than those observed at room temperature. The acceleration in the shortening apparently takes place at the expense

of the period of relaxation or latency. Conversely, in a few rabbit muscles which have been observed at 0°C., 24 hours was required for the attainment of maximum shortening. Such results correlate well with the assumption that the chemical processes responsible for the breakdown of ATP are affected in the usual manner by the environmental temperature.

Since it is well known, as was pointed out by Hoet and Marks ('26), that activity at death has considerable effect on the subsequent course of rigor, electrical stimulation of a muscle would be expected to have a similar effect. Bate-Smith did not find that electrical stimulation hastened the change in the modulus of elasticity. It might be presumed that activity of the muscle before death would reduce, at least somewhat, the amount of ATP, and that such a muscle would then reach the ATP-poor stage (or rigor) more quickly than a normal muscle. In the present studies it was found that muscles subjected to previous tetanization did indeed show an earlier development of shortening.

#### SUMMARY

1. Rat muscles undergoing rigor mortis in a moist chamber consistently shortened as rigor developed.

2. The maximal amount of shortening was maintained for only a few hours, after which the muscle lengthened somewhat, but usually did not return to the original length.

3. The shortening process was affected by the environmental temperature, proceeding very slowly at 0°C., more rapidly at room temperature (25°C.), and most rapidly at 37°C.

4. Muscles which had been subjected to tetanization shortened more quickly than nontetanized muscles.

5. The amount of shortening was similar in all cases.

6. The shortening process is considered to be a very sensitive indicator of the post mortem chemical changes in muscle.



## ACKNOWLEDGMENT

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# THE SURFACE STRUCTURE OF THE GALL BLADDER AND INTESTINAL EPITHELIUM OF MAN AND MONKEY<sup>1</sup>

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SIX FIGURES

The usual textbook statement that the gall bladder is lined by "a single layer of columnar epithelial cells which ordinarily do not possess a striated distal border like those of the intestine but are nevertheless absorptive" (Cowdry, '38) is provocative of further investigation by methods involving the use of phase microscopy and mounting media of different refractive indices.

## METHODS AND MATERIALS

One human and two monkey gall bladders were removed from living anesthetized donors, slit open, and fixed immediately in Zenker-Formol for 10 to 12 hours. The tissues were washed in running tap water for 12 hours, dehydrated in alcohol and infiltrated overnight with 2% low viscosity nitrocellulose, hardened in chloroform (three changes) and infiltrated with 75°C. melting point paraffin. They were sectioned at 1 to 2  $\mu$ , mounted on clean slides without albumen, decerated, run down to 70% alcohol, treated with tincture of iodine followed by sodium thiosulfate and washed in water. They were stored in 70% ethanol. For examination the slides were mounted in media of differing refractive indices as suggested by Crossmon ('49). The phase microscope objective used was

<sup>1</sup>This work was supported in part by a grant-in-aid from the United States Health Service.

an oil immersion Na 1.25 97  $\times$  Dark M (American Optical Company). Photographs were made on Defender 428  $2\frac{1}{4} \times 3\frac{1}{4}$  inch cut film with a wratten B<sub>2</sub> filter.

#### OBSERVATIONS

The tall columnar cells of the epithelium of the gall bladder of man and monkey exhibit fine surface filaments (figs. 1-3). In the monkey the filaments measure from  $0.4\mu$  to  $0.8\mu$  in length and from  $0.1\mu$  to  $0.2\mu$  in width. Their true width may be considerably less. The filaments form an even, narrow fringe (fig. 3). There is no evidence that any of them are fused together, that they are embedded in any "matrix" or that there is an external limiting membrane or cuticle that contains the filaments with it as a "striated border." In the monkey there is no additional structure to be seen in or on the filament. It seems to be a part of the cell membrane. In some cases where the filaments unite with the surface of the cell there is just beneath the cell membrane a light area or vacuole which with other adjacent ones forms a light line. The vacuoles are too large for each to be associated with a single filament (fig. 3). In other instances the filaments project between these vacuoles to a depth somewhat beneath the line of vacuoles. In some instances the cell membrane appears as a black line composed of elliptical beads and the filaments extend between them into the cell substance below. All of these appearances exist and it is not possible at present to regard one as being the definitive condition in the living cell. The terminal bars, found at the surface of the epithelium and between the free edges of neighboring cells are quite prominent (figs. 1 and 3).

The surface of the epithelium in the human gall bladder differed (fig. 2, pl. 1) from that of the monkey. The surface filaments were longer ( $1.5\mu$  to  $.5\mu$ ) although not apparently thicker. The surface of many cells is convex and in these cases the filaments are somewhat separated from each other and there is a knoblike enlargement on the tip of some filaments. These filaments in favorable areas can be seen to pass through the cell membrane and to extend from  $.3\mu$  to  $.5\mu$  be-



low this membrane. This gives the appearance of a zone of somewhat denser cell substance below the cell membrane or a "cap" on top of the cell. In the apical portion of the cell there is a group of opaque, highly refractive granules (C, fig. 2) but there is no discernible association between them and the base of the surface filament.

For the purpose of comparison of the surface filaments of the gall bladder with the surface structure of other portions of the digestive tract, pyloric stomach and ileum of the monkey were fixed, dehydrated and embedded as previously described. The ileum showed the characteristic "striated border" (figs. 4 and 5) of the intestine. As seen by the phase microscope, this border consisted of surface filaments which differed from those of the gall bladder only in that they were longer, more closely spaced on the surface, and showed no evidence of passing through the cell membrane which appeared as a solid black line (B, fig. 4). In some areas there was a light line just beneath the surface of the cell membrane and in other areas it was lacking. The terminal bars showed plainly (C, fig. 4; B, fig. 5).

There were not surface filaments on the cell of the pyloric mucosa. Our observations were limited to the surface and foveolar cells, since we felt the best fixation was effected in this region. The cell surface appeared very smooth. At a small but variable distance beneath the cell surface there was seen an area of specialization which varied widely from cell to cell. In some areas (B, fig. 6) there was a darkened band extending across the top of the cell just below the surface of the cell. Close examination suggested that this dark band was composed of short ( $.3 \pm$ ) rods which align themselves in regular or irregular groups, more or less at right angles to the surface of the cell. In some cases the rodlets appear as irregular groups rather than as a dense band.

#### DISCUSSION

Since the surface filaments cannot be seen with the bright-field microscope and can be seen with the phase microscope, it is evident that the failure of many observers (Cowdry, '38;

Lucas, '32; Maximow and Bloom, '49) to see these filaments is due to their small size and the lack of contrast between them and their surroundings. The rapidity with which the mucosa autolyzes after death might result in the destruction of the surface filaments if fixation is delayed.

We find no morphologic basis for differentiating between the "surface filaments" and such poorly defined entities as "striated border" and "cuticle" (Macklin, '32; Lucas et al., '32). We were unable to resolve structural details such as those reported in the cuticle by Clara ('26). Many of the structures described by Clara are so small as to be theoretically irresolvable with the light microscope. Although we saw structures that might be interpreted as "basal granules" (clear vacuoles) if stained, they were inconstant of occurrence and no direct association between them and the surface filaments could be seen. There was no evidence of any "ground substance" (Clara, '26) in which the filaments were buried in either the gall bladder or intestine. No "border granules" were visible (Clara, '26). Most of these structures are at the extreme range of resolution of the light microscope and it is suggested may be artifacts due to staining or optical diffraction effects.

#### CONCLUSIONS

The epithelial surface of the gall bladder and intestine of man and monkey exhibits a border of "surface filaments" which in the case of the intestine has long been termed the "striated border."

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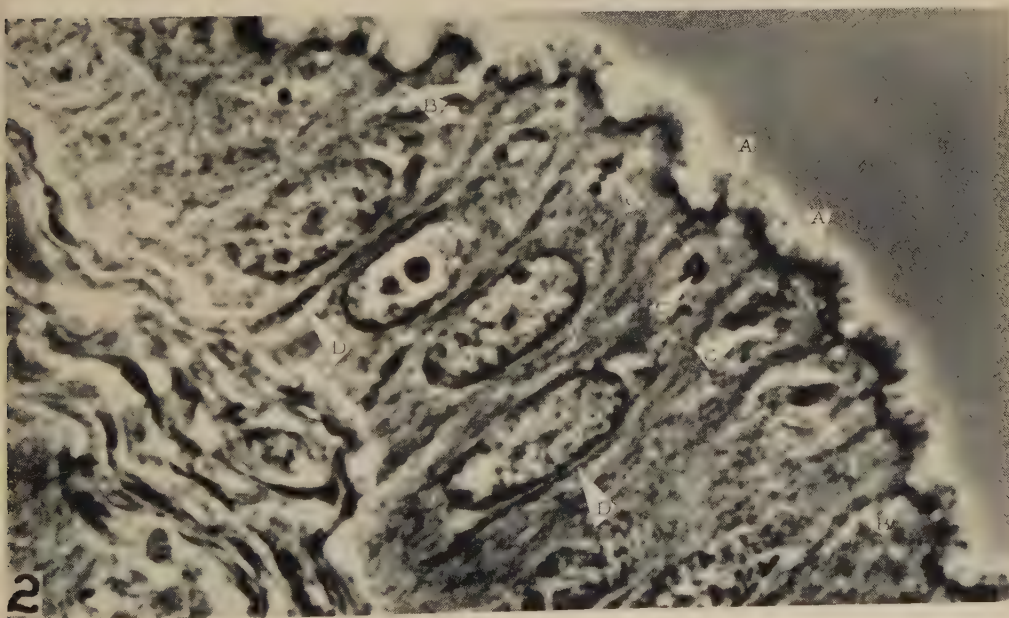
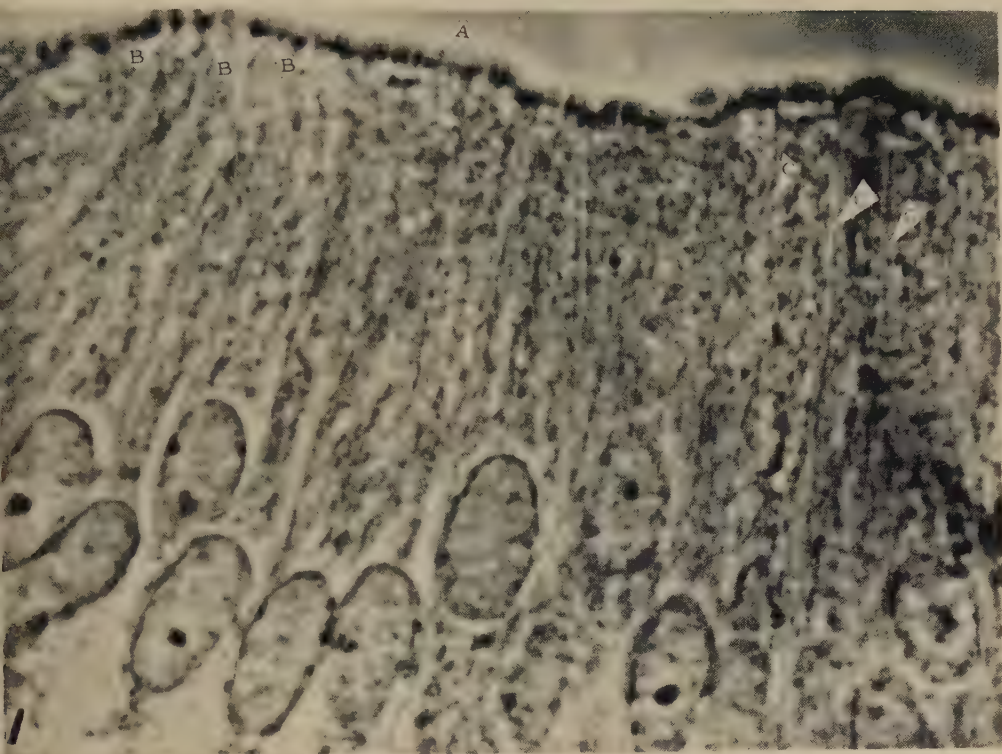
PLATES



## PLATE 1

### EXPLANATION OF FIGURES

- 1 Section of gall bladder epithelium of monkey  $1\mu$  thick, mounted without stain in a medium whose index of refraction was 1.474. Note surface filaments (A), terminal bars (B) and cell membrane (C). Zenker-Formol fixation.  $\times 2332$ .
- 2 Section of gall bladder of man  $1\mu$  thick mounted without stain in a medium whose index of refraction was 1.434. Note surface filaments (A), terminal bars (B), "secretion granules" (C) and cell membranes (D). Zenker-Formol fixation.  $\times 2332$ .



## PLATE 2

### EXPLANATION OF FIGURES

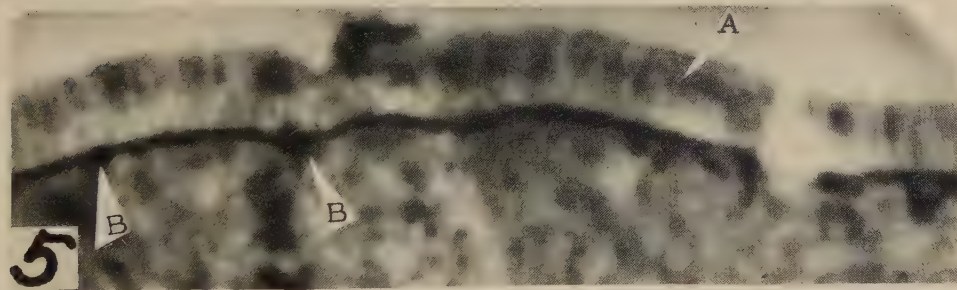
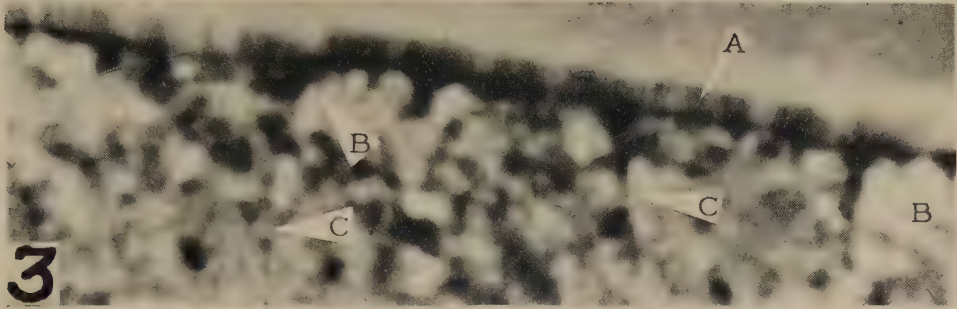
3 Free edge of epithelium of gall bladder of monkey  $1\mu$  thick mounted in medium with a refractive index of 1.434. Note surface filaments (A), terminal bars (B), cell membranes (C) and vacuolated appearance of cytoplasm. Zenker-Formol fixation.  $\times 5000$ .

4 Free edge of epithelium from ileum of monkey  $1\mu$  thick mounted in medium with a refractive index of 1.434. Note surface filaments (A), cell membrane (B) and terminal bar (C). Bouin fixation.  $\times 5000$ .

5 Free edge of epithelium from ileum of monkey  $1\mu$  thick, mounted in medium with refractive index of 1.434. Note "striated border" of surface filaments (A) and terminal bars (B). Bouin fixation.  $\times 5000$ .

6 Free edge of epithelium from pyloric stomach of monkey  $1\mu$  thick mounted in medium with refractive index of 1.422. Note smooth surface of cell composed of homogenous cell substances (A) and dense area beneath cell membrane (B). Zenker-Formol fixation.  $\times 5000$ .







## SUBCLAVIAN ORIGIN OF BRONCHIAL ARTERIES

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### FIVE FIGURES

The variability of the bronchial arteries is mentioned by a number of writers but details of the topography of these variations are difficult to come by, although "knowledge of their variable origins and distribution would provide a practical addition to the operator's armamentarium" (Cauldwell et al., '48).

### DESCRIPTION OF CASE

Male dissecting room body, *aet.* 66; certified cause of death: arteriosclerosis. The right and left bronchial arteries originated from a single stem which arose in common with the left superior intercostal artery from the left subclavian (fig. 1). This common "intercostobronchial trunk" was encircled by the ansa subclavia, and after a course of about one inch divided into its intercostal and bronchial branches, the former vessel supplying the first and second intercostal spaces. The bronchial trunk passed downwards behind the stellate ganglion (fig. 2), being partially embedded in its posterior surface. It then coursed obliquely downwards and medially behind the left subclavian artery to reach the front of the oesophagus. Two main oesophageal branches were given off; the larger and proximal behind the subclavian artery, the smaller and distal in the tracheo-oesophageal groove. The common bronchial artery then continued downwards behind the aortic arch and behind the left recurrent laryngeal nerve, giving a branch to a superior tracheobronchial lymph gland situated above the left bronchus (fig. 3). The artery then passed to the front of the trachea a short distance above the tracheal bifurcation,

and at the tracheal crotch (carina) divided into a larger right and a smaller left bronchial artery; these branches being distributed along the antero-inferior aspect of the bronchi. The right artery accompanied the hyparterial bronchus.

#### SUBCLAVIAN ORIGINS

Bronchial arteries rarely arise from the subclavian artery. Only about 12 cases appear to have been published and these generally with no topographical details or illustrations. A few instances of origin from the internal mammary or superior intercostal have also been published, and Quain's (1844) case is a combination of these two anomalous derivations; two common trunks were present, one from the right internal mammary to the fronts of the bronchi, and the other from the right superior intercostal, passing behind the right subclavian artery to the backs of the bronchi (the latter branch is incorrectly described as arising "on the left" side by Cauldwell et al.). From the illustration in Quain the superior intercostal vessel appears to be considerably smaller than the bronchial artery and from the point of view of size (as opposed to an embryological interpretation) is merely a branch of the latter. We follow Cauldwell and his co-workers, therefore, in using the term "intercostobronchial" to "obviate artificial and confusing distinction between parent stem and branch." These authors found no specimen in 150 cases in which major bronchial arteries were derived from the costocervical trunk, with the exception of a case of situs inversus viscerum where the left intercostobronchial artery supplied the first three intercostal spaces.

We have avoided the term "accessory bronchial artery" in our case because, owing to the late stage of the routine dissection at which the anomaly and that of the other cases we describe later were noticed, we were unable to determine whether or not other bronchial arteries were present. It is to be observed, however, that the right branch accompanies



the hyparterial bronchus. Cauldwell and his colleagues give the following terminological information in a footnote: "Meckel (1832) describes branches from the subclavian or corresponding internal mammary arteries as 'superior bronchial arteries.' Other authors generally refer to these as

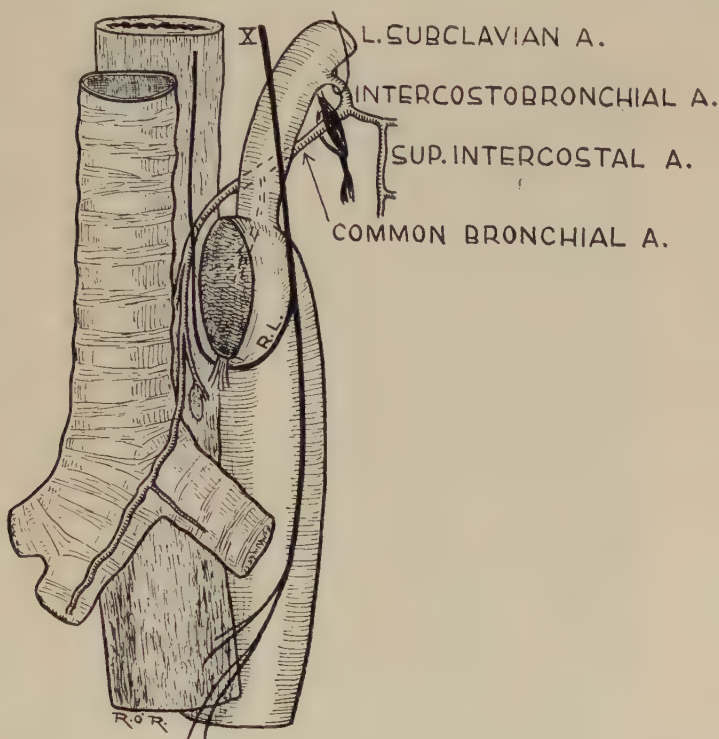


Fig. 1 Anterior aspect of trachea, oesophagus, and aorta, showing course of anomalous bronchial artery. For details see text.

'accessory bronchial' or 'anterior bronchial' arteries in contrast to 'posterior' or 'inferior' vessels; the latter terms referring to the usual arteries of aortic or intercostal origins." Rauber-Kopsch ('29) use the term "Aa bronchiales superiores" for branches from the concavity of the aortic arch.

In the following published cases bronchial vessels were derived from the left subclavian artery:

1. Fickel (1743, cited by Cauldwell et al.). Common bronchial artery to both lungs from subclavian, probably of left side.

2. Meyer (1857). Left bronchial artery from the left subclavian, right bronchial artery from the concavity of the aortic arch; both go behind the bronchi. Not illustrated.

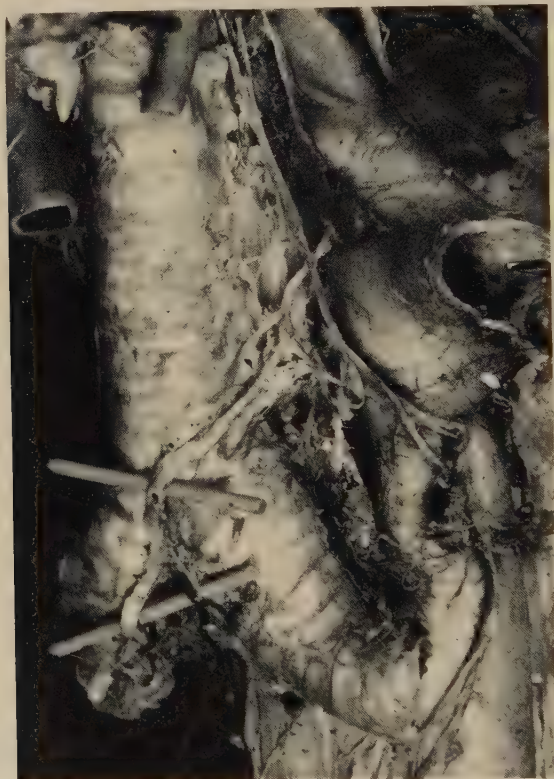


Fig. 2 (a) Photograph of left aspect viewed from below and behind. The superior intercostal artery is held up by a pin.

3. Turner (1862). A short thick pouch-like trunk arose (above the right bronchus) from the left side of the descending part of the arch of a right-sided aorta. It passed transversely to the left behind the trachea and oesophagus, at the level of the disc between the third and 4th thoracic vertebrae. At the left margin of the oesophagus it joined the obliterated

ductus arteriosus and at the point of junction the left subclavian artery arose. "From the same pouch-like trunk a small bronchial artery for the left lung proceeded." The bronchial vessel is not illustrated.

4. Hewitt ('30). From the postero-medial aspect of the left subclavian, passing over the aortic arch to the back of the left bronchus. Not illustrated.

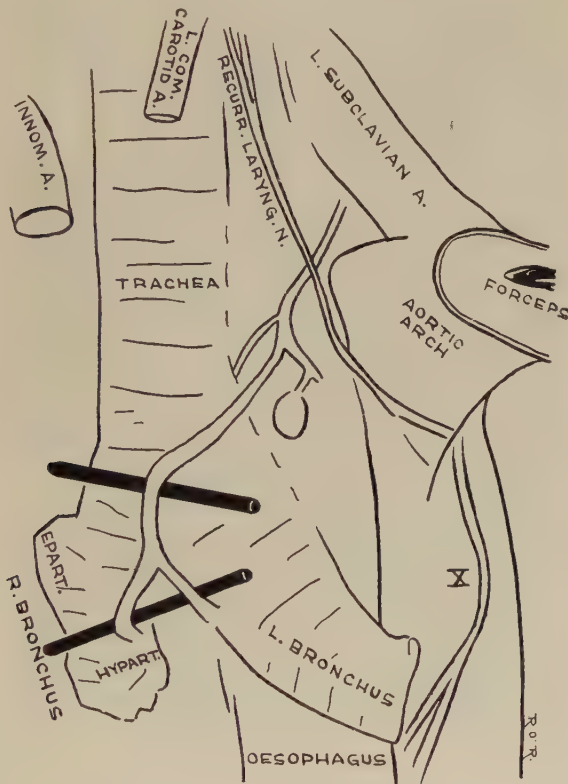


Fig. 2 (b) Key diagram to figure 2 (a).

5. Schneider ('34). From the posterior side of the left subclavian, passing to the tracheal bifurcation and dividing to supply the right bronchus. Not illustrated.

6. Cauldwell et al. ('48). From the left subclavian (medial to the internal mammary) to the right and left upper lobe bronchii. Not illustrated.

7. Cauldwell et al. ('48). From the left subclavian to the left upper lobe bronchus. From the illustration of this case (fig. 5, p. 403, of their paper) this artery can be seen to pass in front of the aortic arch and on the left side of the left recurrent laryngeal nerve, although its precise relationship



Fig. 3 (a) Photograph of anterior aspect. The cut end of the aortic arch has been retracted by forceps to the left hand side.

to the bronchus is not clear; middle and inferior left bronchial arteries arise from the thoracic aorta. These writers found only three subclavian origins (one right and two left) in 150 subjects (2%) and in all three instances other bronchial arteries of aortic derivation were present. They list Ruysch,



Quain, Luschka, and Poupardin as authors who described bronchial arteries of superior intercostal origin.

#### PREBRONCHIAL COURSE

The silence of textbooks concerning the existence of arteries on the fronts of the bronchi is noticeable, especially as illustrations of such an arrangement are given in a number of manuals. Cauldwell and his co-authors trace a number of diagrams showing bronchial arteries arising from the concavity

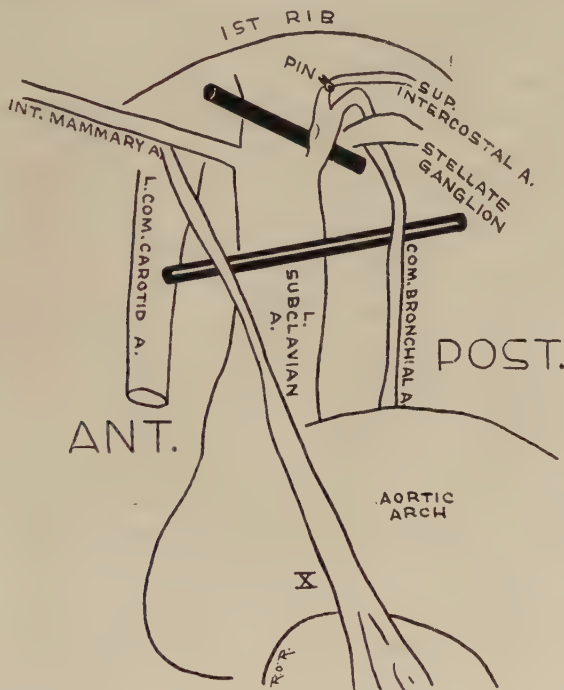


Fig. 3 (b) Key diagram to figure 3 (a).

of the aortic arch and descending thoracic aorta and lying on the front of the trachea and bronchi back to Tiedemann's *Tabularum Arteriarum*, 1822 (e.g., Quain, 1892; Rauber-Kopsch, '29; Morris, '42). We have also observed other illustrations displaying arteries on the fronts of the bronchi, e.g., Toldt, '28; Sobotta, '32; Grant, '47; these appear to be com-

mon trunks from the aortic arch or descending thoracic aorta and supplying right and left bronchi. Boyden's ('45) case is referred to later.

It is difficult to estimate the frequency of arteries on the fronts of the bronchi. Cauldwell and his co-writers state that vessels from the anterior surface of the thoracic aorta may cross anterior to the tracheal bifurcation and continue along the caudal border of the right main bronchus; and of 39 (26%) of their 150 cases in which the right and left bronchial arteries arose from a common stem, 8 passed anterior to the trachea or tracheal bifurcation to reach the right bronchus. That prebronchial vessels are perhaps more frequent than is generally supposed may be inferred also from Kent and Blades ('42) who find that while the bronchial arteries "are said to be found overlying the membranous posterior wall of the main bronchus, our observations indicate that there is great variation in their relationship to the main bronchus and to the branch bronchi."

Prebronchial vessels belonging to the pulmonary system have been recorded by Adachi ('28) who describes an "A praebronchialis" arising from the left pulmonary artery.

In an examination of 25 subjects (18 dissecting room bodies and 7 autopsy cases) we encountered two dissecting room instances of arteries on the fronts of the bronchi, in addition to the case of subclavian origin already described. Furthermore, another instance was found among a series of prosecutions. These cases are described in the following notes:

(a) Male, *aet.* 65. Certified cause of death: Friedrich's ataxia. A common bronchial artery arose from the right anterior aspect of the commencement of the descending thoracic aorta and passed to the front of the trachea immediately above the tracheal crotch. It divided into two equal-sized branches, distributed along the antero-inferior aspect of the right and left bronchi. The artery was inferior to the left recurrent laryngeal nerve.

(b) Male, *aet.* 80. Certified cause of death: arteriosclerosis. A common bronchial artery arose from the right posterior

aspect of the aortic arch, and looped over the left recurrent laryngeal nerve. Its relations and distribution to the trachea and bronchi were precisely as in the previous case.

(c) Prosection. A left bronchial artery arose by a common trunk with the artery of the second left intercostal space from the upper part of the left side of the aortic arch. It passed downwards on the left side of the aortic arch, crossed behind the left vagus below the origin of the recurrent laryngeal nerve, and reached the antero-superior aspect of the left bronchus, where its branches became lost. This last case is unusual in that left bronchial arteries rarely arise from the left side of the aorta and seldom arise in common with an intercostal vessel, according to Cauldwell et al. These authors found one case in which a left bronchial artery arose from the antero-superior aspect of the aortic arch, immediately caudal to the roots of the innominate and left common carotid arteries, and coursed anterior to the trachea to reach the left bronchus.

#### COMMON STEM ORIGINS

In this section we refer to the origin of right and left bronchial arteries (i.e., to the right and left lungs) or accessory bronchial arteries from a common stem. In 39 (26%) of the 150 cases examined by Cauldwell and his co-workers the right and left bronchial arteries arose from a common stem. Of 21 cases where the bronchial arteries came from the concavity of the aortic arch, 4 instances of a common stem providing branches to the right and left bronchi were encountered by these writers. According to the same authors the right and left bronchial arteries usually originate from a common trunk when there are multiple right bronchial arteries.

A number of the cases illustrated in the standard anatomical atlases and mentioned previously are instances of common stem origin, as is the case figured by Quain (1844) and as Haller's specimen is said to have been. More recently, in a case featured by Boyden ('45) the bronchial arteries arose from a common stem from the aortic arch, the left passing anterior to the left bronchus, and the right coursing behind

the left bronchus to the front of the oesophagus to reach the back of the right bronchus.

We encountered three instances of common stem origin, as described above.

#### EMBRYOLOGY

No information is available concerning the embryology of the bronchial arteries as such (Keibel and Mall, '12), but in view of their close association with the intercostal vessels in the adult a consideration of the development of the latter is in place.

The paired dorsal intersegmental arteries of the embryo, which arise from the dorsal aortae before these have fused, are associated primarily with the neural tube. They form the spinal branches of the adult intercostal arteries and their secondary but larger body-wall branches are the definitive intercostal and lumbar arteries themselves. In the cervical region series of longitudinal anastomotic channels between the dorsal intersegmentals (postcostal anastomosis) gradually replace the latter and form the vertebral arteries; these ultimately arise from the 6th or 7th or 8th dorsal intersegmentals, one set of which enlarges to constitute the subclavian. The two or three intersegmental arteries immediately caudal to the subclavian form a precostal anastomosis (superior intercostal artery) with the latter vessel and supply generally the first two intercostal spaces. Mall considered that "the descent of the heart into the thorax on the inside with the descent of the arm over the clavicle on the outside of the body causes great tension on the upper intercostal arteries, and favors the new formation of blood-vessels in a more direct line," and thus the superior intercostal is a secondary and direct artery from the subclavian (Keibel and Mall, '12).

Walmsley ('30) looks upon the bronchial arteries as continuations of splanchnic vessels to diverticula of the gut, and so "the usual origin of the right vessel from the first aortic intercostal artery must result from the enlargement of an anastomosis between a splanchnic vessel and the first part of an intersegmental somatic vessel; the anastomosis is much



less frequently developed on the left side . . . ” Cauldwell et al. ('48) suggest “secondary development of the pulmonary splanchnic arteries from the lower cervical and upper thoracic dorsal segmental (intercostal), and from the ventral aspect of the aorta during the course of, and subsequent to, the descent of the lungs into the future thoracic cavity.” These authors also stress the close proximity of the aortic arch to the left bronchus and of the intercostal arteries to the right bronchus, permitting easy access of visceral branches from these vessels to the corresponding bronchi. It seems possible, although we are not aware of any direct evidence for such a supposition, that a succession of bronchial vessels arise in embryonic life, somewhat after the manner of the renal vascularization, and that usually only the lowest vessels remain; accessory bronchial vessels might then be a persistence of one or more of the higher vessels. A multiple embryonic vascularization would be in keeping with the varied relationships of anomalous bronchial arteries to the left recurrent laryngeal nerve.

The pathological elaboration of anastomoses must also be kept in mind. The bronchial arterial circulation of the dog increases tremendously following ligation of the pulmonary trunk, and great enlargement of the bronchial and their anastomoses with the pulmonary arteries occurs in chronic pulmonary disease in man (Liebow et al., '47).

It seems possible that in the present case of subclavian origin a splanchnic-intersegmental anastomosis occurred at a high level and the bronchial and superior intercostal vessels so associated retained their connection throughout development as an intercostobronchial trunk, the bronchial part of which elongated with the descending thoracic viscera. If this anastomosis took place before the precostal anastomosis replaced the immediately post-subclavian intersegmentals, the largely dorsal relationship of the bronchial artery to the sympathetic trunk in our case is in accord with the state-

ment that whereas the first two intercostals pass dorsal to the sympathetic originally they pass ventral to it when the superior intercostal is formed (Keibel and Mall, '12).

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## FURTHER EXPLORATION OF THE $\text{HIO}_4$ -SCHIFF REACTION WITH REMARKS ON ITS SIGNIFICANCE

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Repeated statements have appeared in recent literature that the periodic acid Schiff reaction described by McManus ('46), by me independently ('47a, b), and by Hotchkiss ('48), indicates in animal tissues the presence of a mucopolysaccharide.

Glick ('49) simply characterized the reaction as a general one for the visualization of polysaccharides, and notes that in animal tissues the chief substances which react are "glycogen, mucin, mucoproteins and presumably hyaluronic acid and chitin." "Cerebrosides, if present, would be expected to react."

Pearse ('49a, b) notes that besides glycogen and certain sugar containing lipoids, "in practice, however, bearing in mind these objections, a positive result with a negative control indicates mucopolysaccharide, mucoprotein or glycoprotein. He recorded positive reactions in  $\beta$  granules of the anterior hypophysis, in Russell bodies, in certain globules and vesicles in the trophoblast layer of the placenta and in Langhans cells of chorionoeplithelioma and the granules of the tissue mast cells. He argued from the reaction that the  $\beta$  granules contain the mucoprotein pituitary gonadotropic hormone, that the chorionic gonadotropin, also a mucoprotein, has been localized in the trophoblast globules, that the Russell bodies are probably mucoprotein, and that the positive reaction of the mast cell granule supports its presumed identity with the mucopolysaccharide heparin.

Davenport ('50) writing of the periodic acid Schiff reaction of argyrophil reticulum speaks also of a mucopolysaccharide or mucoprotein nature of this substance.

Also Gersh ('49) concludes that the portion of the Golgi apparatus that he demonstrated by the periodic acid Schiff reaction was to be classed as a carbohydrate-protein complex.

While McManus ('46) simply recorded the positive reactions of mucin, reticulum, connective tissue fibers and other substances, by 1948 ('48a, b) he was referring to glycogen, mucin, basement membrane, reticulin, thyroid and pituitary colloid, the granules of some anterior hypophysis cells, etc., as "carbohydrates of tissues." Again ('48b) he suggests that the renal glomerular basement membrane contains a mucoprotein, but recalls that the amino acids serine, threonine and hydroxylysine as well as glycolipid might react.

Much of this thinking follows the basic chemical work of Hotchkiss ('48) whose methods were known to McManus, Glick, Gersh and perhaps others before their publication. Hotchkiss applied the Schiff aldehyde reagent to a large number of more or less pure substances after oxidizing with periodic acid in spot tests. Many of these were of polysaccharide nature, some were proteins or amino acids.

It is my purpose here to record positive Schiff reactions after oxidation by periodic acid in a number of additional tissue elements.

After the action of relatively slightly acid or neutral aqueous fixatives, zymogen granules of pancreas and serous salivary gland and the Paneth cell granules of the intestinal crypts of Lieberkühn are generally well preserved and stain in varying shades of red by an azure A, eosin B technic based on that published on page 84 of my *Histopathologic Technic* ('48).

In man the pancreatic zymogen granules stain brilliantly red purple after ordinary 10% formalin fixation. This confirms the observation of Hartz ('48) on Bouin-sublimite and sublimite formalin material from human infants. Hartz made no deductions about mucoprotein nature of these granules. In



rats, guinea pigs, mice and dogs, pancreatic zymogen granules did not react to this method, while in rabbits they gave a red to orange red color, especially after bichromate formalin fixatives. The Casella and Bauer (Lillie, '48) reactions are negative in all 5 species.

Paneth cell granules seem less resistant to acid fixatives than pancreatic or salivary zymogen granules. Pearse ('49b) recorded them as reacting faintly or not at all to the periodic acid Schiff test, but does not record either the species examined or the fixation used. In mice we found a rather weak reaction, absent altogether in some mice, and in some levels of the intestine in others. With our picric acid counterstain they colored light orange or brownish yellow. In guinea pigs granules stained orange to pink in most sections where red or pink granules were demonstrable by azure eosin. In rats Paneth cell granules were usually quite well stained in orange red tints. The picric acid counter stain demonstrates the oxyphilia of the granules and by altering their color from the red purple of the aldehyde reaction to orange red, aids in differentiating them from the still red purple mucin and mucigen granules. Some rabbits gave strong periodic acid Schiff reactions of their Paneth cell granules, others a negative, but in the latter few or no Paneth granules were found with azure eosin stains. In one dog no Paneth cell granules were found with three fixations that were satisfactory in other species. Malt diastase digestion tests did not alter the  $\text{HIO}_4$ -Schiff reaction of Paneth granules, and by removing the cytoplasmic basophilia of the gland cells, actually made them more conspicuous with azure eosin stains. Bauer and Casella oxidation Schiff reactions were negative in all 5 species.

Salivary gland zymogen granules also fail to react to the permanganate and chromic acid oxidation Schiff tests. Strongly positive reactions were given with the periodic acid Schiff tests in 7 human, 1 dog, 2 rabbit, 4 rat and 3 guinea pig salivary glands. Formalin fixation was used in all, Kose's or Möller's bichromate formalin in all the animals, as well as a variety of other fixatives. These granules seemed to

resist acid fixatives somewhat better than those of the pancreas. They are generally much less eosinophilic than pancreatic zymogen or Paneth granules. Both this eosinophilia and the periodic acid Schiff reaction are unaltered by malt diastase digestion.

The zymogen granules of the gastric chief cells are most often recognized as unstained bodies in the acinar end of the intensely basophilic cytoplasm. They tinge yellow with picric acid and may be pale blue to blue green with acid thionin (at pH 5-6 but not at pH 3) and, in some guinea pigs pink or even red with azure eosin. In rats and mice this stain occasionally gives a pink color but not in the dog or the rabbits. They resist malt diastase digestion. They fail to react to Schiff reagent after oxidation by potassium permanganate, chromic acid or periodic acid. In guinea pigs the orange pink eosinophilic granules may be seen to be closely associated with or even to enclose deep red purple mucigen granules when the azure A eosin B technic is used as a counterstain to the periodic acid Schiff test.

The chromaffin substance of the adrenal medulla is stained yellowish green to green after unacidified bichromate formaldehyde fixatives, less definitely and variably so with acetic bichromate fixatives. After the periodic acid Schiff sequence the same material is stained deep purplish red with the first group of fixatives, unstained to pink with the acid chromate fixatives, and unstained with such non-chromate fixatives as neutral aqueous and alcoholic formalin, acetic alcohol formalin, Gendre's, Bouin's and Carnoy's fluids. Without periodic acid treatment chromaffin is Schiff negative. Also with Bauer's method it fails to react. Permanganate oxidation gave in one series good to strong Schiff reactions of the chromaffin substance after chromate fixation, but negative reactions in later series, and weak, dubious or negative reactions with other fixations in all series. Schmorl's ferric chloride potassium ferricyanide mixture colored medulla cells deep blue green after unacidified bichromate formalin fixations, and gave the same diffuse pale green as the rest of the tissue

after non-chromate fixatives. Fixation of fresh rabbit adrenal in 10 parts of 27% ferric chloride and 90 parts alcohol failed to preserve chromaffin. Medulla cell cytoplasm blackened moderately with Masson's argentaffin cell method after bichromate formalin fixations and not after non-chromate fixatives. The foregoing is based on guinea pig and rat adrenals. A few human adrenals fixed in Orth's fluid gave the same reactions.

It may be noted at this point that regardless of fixation method we have not been able to identify gastro-intestinal argentaffin cells with the periodic acid, chromic acid or potassium permanganate oxidation Schiff techniques.

The cortex cells of the reticular zone of the guinea pig adrenal often contain fine granules of light to deep brown pigment, and similar pigment, usually of deeper brown color in the same adrenal, is seen in phagocytes lining the capillaries of the fascicular zone. This pigment often colors red with Schiff's reagent after periodic acid oxidation, though some granules remain brown, and various transitions of color from brown to purplish red occur. A few granules also give the Casella permanganate Schiff reaction, though most do not, and often some of the denser pigment in the phagocytes tinges pink with Bauer's chromic acid leucofuchsin method. Diastase digestion does not decrease the periodic acid Schiff reaction. Like the human lipofuscins these pigment granules are usually partially to completely blackened by diammine silver hydroxide (Masson's argentaffin technic). They retain more or less red coloration with Mallory's hemofuscin technic, but are seldom acid alcohol fast with the Ziehl Neelsen method. They usually color green with azure A and orange with safranin, though the intensity of this basophilia is quite variable. Without oxidation the granules are Schiff negative. The foregoing reactions are not influenced by the method of fixation. After non-chromate containing fixatives they generally reduce Schmorr's ferric chloride, potassium ferricyanide "lipofuscin" reagent, coloring a more or less greenish blue. After chromate fixations: Orth, Kose, Möller-Regaud, Spuler, Zenker, Tellyesniczky, this reaction is abolished, indicating that

the reducing groups are destroyed by potassium bichromate oxidation with or without acetic acid, formaldehyde or mercuric salts. From the persistence of the pigment after fixation in Carnoy's acetic chloroform alcohol as well as in the formaldehyde alcohols with or without acetic or picric acid it would appear that the pigment is none of the more readily soluble lipid complexes.

Lipofuscin in cells of the reticular zone of the human adrenal cortex also gave a positive periodic acid Schiff reaction and a positive Schmorl ferric ferricyanide reaction. The latter was abolished by oxidation of sections for one hour with 5% chromic acid ( $\text{CrO}_3$ ) or for 20 minutes with 1%  $\text{KMnO}_4$ , with and without a following 5 minute reduction in 1% oxalic acid. The reaction was not affected by oxidation in 3%  $\text{H}_2\text{O}_2$ , in 1%  $\text{NaIO}_3$  or for one hour in 5%  $\text{K}_2\text{Cr}_2\text{O}_7$ . Ten minute oxidation by 0.8%  $\text{KIO}_4$  in 0.3%  $\text{HNO}_3$  abolished the ferric chloride potassium ferricyanide reduction in one series of tests on Carnoy and formalin fixed human adrenals, while in another series oxidized with 1%  $\text{Na}_3\text{IO}_5$  in 0.5%  $\text{HNO}_3$ , the reaction persisted. This reaction was not given by pigment in parallel blocks of the same adrenals which had been fixed in Orth's fluid.

The human adrenal lipofuscin pigment usually blackens with the Gomori-Burtner methenamine silver argentaffin cell technic (Burtner and Lillie, '49) before reticulin does. Pre-oxidation by 0.25% potassium permanganate for 1 hour, followed by a 2 minute bleach in 5% oxalic acid, or by 5% chromic anhydride for 1 hour or by a 0.3%  $\text{HNO}_3$ , 0.8%  $\text{KIO}_4$  solution for 10 minutes did not prevent the reduction of silver. Treatment for 10 minutes in 1% ferric chloride regularly prevented the reduction of methenamine silver by this lipofuscin, although it enhanced the blackening of reticulum. Reticulum was usually not blackened or only partially or slightly so in the  $3\frac{1}{2}$  hour exposure given when no preliminary oxidation was used. Ferric chloride pre-treatment regularly gave quite complete blackening of reticulum, chromic acid, periodic acid and potassium permanganate less regularly and less complete.



Thus it would appear that ferric chloride blocks or destroys the silver reducing groups in the "lipofuscin" though it enhances the reducing capacity of reticulum.

Hueck ('21) has noted a brown granular lipofuscin pigment in the reticular zone of the adrenal and in aging lutein cells of the ovary. This pigment he regards as closer to the melanins than some other lipofuscins. He states that it does not reduce "silver" though Schmorl ('28) notes from Staemmler's work that lipofuscin blackens with diammine silver hydroxide.

From the location and the reactions described, it appears probable that the pigment described in guinea pig adrenals is at least similar to if not identical with human adrenal lipofuscin. The latter on a few trials gave no hemofuscin stain, but this was weak in many of the guinea pig adrenals. Neither pigment stained with oil blue N. It is to be noted that granular brown pigment is often found in cells of the reticular zone in man untinged by red even in frozen sections stained with oil red O for fats.

There remain two, or possibly a single substance giving a positive  $\text{HIO}_4$ -Schiff reaction on which I wish to report at this time. These are the so-called melanin of human melanosis coli and an apparently analogous, but sometimes iron positive pigment occurring more or less plentifully as fine to coarse, oval and rounded granules in large phagocytes in the mucosa of the small and large intestine of guinea pigs. The human material comprised 10 appendices derived from my ('31) previous studies on melanosis of the appendix. The guinea pig material comprised sections of various levels of small and large intestine fixed in parallel in 4 to 6 different fixing solutions designed to test protein granule solubilities during fixation. The intestines of 6 guinea pigs presented large enough amounts of this pigment to warrant tabulation. Fixations included neutral and acid, aqueous and alcoholic formalin, neutral and acid alcohol, picric acid mixtures with and without formalin, mercuric chloride mixtures with acetic acid or calcium acetate, with and without formalin, Bouin, Zenker, Zenker formalin.

Results for the two pigments are presented as follows:

*Reactions of melanosis appendix pigment in man, and of guinea pig intestinal pigment*

| REACTION           | DIRECT<br>SCHIFF | HIO <sub>4</sub><br>SCHIFF | CrO <sub>3</sub><br>SCHIFF | KMnO <sub>4</sub><br>SCHIFF | FERRIC<br>FERRICY. | POTASS.<br>FERROCY. |
|--------------------|------------------|----------------------------|----------------------------|-----------------------------|--------------------|---------------------|
| Human<br>melanosis | —                | ++                         | ±                          | — to ±                      | ++                 | —                   |
| Guinea<br>pig      |                  | +                          | — to ±                     | — > ±                       | +                  | Var. +<br>and —     |

| REACTION           | ARGENTAFFIN |      | AzA<br>EoB | pH <sub>4</sub><br>THION. | OIL<br>BLUE | MYELIN | HEMOFUSCIN |
|--------------------|-------------|------|------------|---------------------------|-------------|--------|------------|
|                    | Masson      | G.B. |            |                           |             |        |            |
| Human<br>melanosis |             | +    | green      | blue<br>green             | —           | —      | ±          |
| Guinea<br>pig      | ± to +      | +    | green      | blue<br>green             |             |        |            |

The human pigment was insoluble on 24 hour exposure to 10% acetic acid, 10% hydrochloric acid (1.25 N) and 5% borax solutions. It gave a negative Gmelin reaction. Oxidation for one hour in 5% potassium bichromate, in 5% chromic anhydride or in 1% hydrogen peroxide or for 10 minutes in 0.8% potassium periodate with 0.3% nitric acid or in 0.8% sodium iodate with 0.3% nitric acid did not inhibit the ferric ferri cyanide reduction test, but oxidation for one hour in 0.25% potassium permanganate followed by a one minute bleach in 5% oxalic acid did destroy the capacity to reduce ferric ferri cyanide. In regard to the negative ferro cyanide reaction, it is to be remembered that such a pigmentation of the colon, yielding a positive iron reaction, would automatically be excluded from melanosis coli and classed probably as pseudomelanosis.

Cutaneous melanin in the guinea pig gives no reactions by the HIO<sub>4</sub>, CrO<sub>3</sub> or KMnO<sub>4</sub> oxidation Schiff technics, it tinges green with azure A and with thionin, it reduces Fontana's

diammine silver by hydroxide in Masson's argentaffin method, it is Gram negative by the Weigert technic, and it fails to reduce ferric ferriocyanide in the Schmorl-Laskey technic. It was bleached by  $\text{KMnO}_4$  after certain alcoholic fixations only.

Human splenic hemosiderin did not give the periodic acid Schiff reaction. Inasmuch as Wislocki, Rheingold and Dempsey ('49), Pearse ('49a, b) and Smith ('50) have each noted positive periodic acid Schiff reactions of mast cell granules, and previous workers with the method have not reported this finding, it seems proper to detail our own past experience with this subject. In the course of then current studies on the reactions of the mucins, we noted some cells in the connective tissues with fine to medium pink to red granules. These were usually far fewer than the metachromatically basophil mast cells seen in the parallel thionin, azure eosin and iron hematoxylin, fast green, safranin preparations, and we hesitated to identify them as mast cells, more especially as in some rat and mouse stomach sections we found no cells with Schiff positive granules, though fairly numerous mast cells were seen with the metachromatic basic dyes.

In view of the existing doubt on the reaction of mast cell granules, a number of thionin stained sections of human appendix, rat omentum and salivary gland and mouse stomach were explored and in selected areas the position of all the mast cells was charted, with their relation to surrounding structures. The sections were then demounted and decolorized with acid alcohol, and the periodic acid Schiff reaction performed, using 0.8%  $\text{KIO}_4$  in 0.3%  $\text{HNO}_3$  for 10 minutes as oxidant. After the Schiff bath and sulfite washes the sections were counterstained with Weigert's iron chloride hematoxylin and picric acid (Lillie, '48, p. 189). The same areas were then recharted and the position of all Schiff positive cells plotted. Then the sections were again demounted and counterstained with methylene green (C.I. no. 924: mononitromethylene blue). This dye gives a rather light green stain to nuclei and basophil cytoplasm, and a deep slightly greenish blue or blue black color to mast cell granules. It

was chosen as giving a color as different from the red purple to red of the Schiff reaction as possible.

Summarizing the results of these explorations, we found that many of the previously located mast cells could not be found at all in the second,  $\text{HIO}_4$ -Schiff charting, and were again located with blue black granules in the final re-charting. In some of the chartings all cells reacted in this manner. In other preparations some of the definitely re-located mast cells were found to present fine or medium pink or purplish pink granules or a diffuse pink coloration of the cytoplasm in the Schiff reaction charting. There were also found some cells with similar granular and non-granular Schiff positive coloration, which had not been charted with the thionin stain. On re-staining with methylene green, all or nearly all the cells originally stained with thionin were now found to possess deep greenish blue granules, while a number of other cells which had periodic Schiff positive cytoplasm or granules at the second charting still possessed pink or purplish red cytoplasm or granules.

In personal correspondence C. Smith tells me that some mouse mast cells contain periodic Schiff positive material and others do not, in agreement with the foregoing findings. She suggests that the  $\text{HIO}_4$ -Schiff reaction may be a phenomenon associated with the age of the cell. In our material it was often large mast cells that gave the reaction, but not always. Whether the Schiff positive cells without metachromatic granules are to be regarded as still another developmental phase in the life cycle of the mast cell is at this writing uncertain. It may be that some mast cells share with certain other connective tissue cells the property of possessing diffuse or finely or coarsely granular periodic Schiff positive material.

The finding that many, or perhaps most mast cell granules do not give the periodic acid Schiff reaction would appear to argue against a mucopolysaccharide nature of their major component. That this substance is acid in nature is strongly indicated from its avidity for basic dyes. The phenomenon of metachromasia has been attributed to various factors, such



as polymerization of dye, and metachromatic staining of tissues has been attributed to the presence in them of polysaccharide sulfuric acid esters of the general type of chondroitin and mucoitin sulfuric acids. Perhaps a simpler explanation of metachromasia would derive from the parallel in inorganic chemistry, that the salt solutions of a given base with some acids may differ sharply in color from those with other acids. Further, solid compounds of a given base with various acids may differ sharply in color. Similarity in color of two salts of a given base, such as thionin, is not particularly good evidence of the close relationship of the acid radicals involved. Hence the production of a "metachromatic" color with thionin, toluidin blue, azure A, safranin O and methylene green does not necessarily prove that all other substances producing a "metachromatic" stain are polysaccharide sulfuric acid ester complexes, just because cartilage matrix and some of the mucins readily give this reaction and contain complexes of that nature. Moreover, it should be recalled that the "metachromatic" colors given by various substances may often vary as much among themselves as some of them do from the so-called "orthochromatic" color. The metachromasia of thionin varies all the way from slightly reddish violet to nearly pure crimson.

#### DISCUSSION

Thus the list of substances and anatomical elements giving the  $\text{HIO}_4$ -Schiff reaction now includes certain zymogen granules, which from their ready solubility in dilute acetic acid, have generally been considered albuminous in nature, the chromaffin substance of the adrenal, which is considered to be an oxidation product of adrenalin, the so-called "lipofuscin" pigment of the adrenal and the so-called "melanin" of melanosis coli and an analogous intestinal pigment in guinea pigs in addition to the hexose polysaccharides glycogen, starch and cellulose, the glycoprotein amyloid, the proteins collagen, gelatin and reticulin, sometimes coagulated plasma, serous exudates and fibrin, Russell and Kurloff bodies,

corpora amylacea of the brain and cord, the mucopolysaccharides which contain mucoitin and and chondroitin sulfuric acids, according to some also the hyaluronic acid mucins, ovarian follicular fluid and the zona pellucida of the ovum, the choline deficiency cirrhosis pigment ceroid in rats, the vitamin E deficiency pigment (Elftman, Kaunitz and Slanetz, '49), hypophyseal and thyroid colloids, the  $\beta$  granules of the anterior hypophysis, the galactolipin kerafin (Morrison and Hack, '49), the chitins of yeasts and molds and of some bacteria, the chitins of some helminth ova notably *Schistosoma*, but not those of round worms and tapeworms, which have been previously recorded.

None of the writers intend, I believe, to include the hexose polysaccharides themselves as "mucopolysaccharides," and the galactolipins are specifically excepted by some. Collagen, which is converted to gelatin on boiling, contains notable amounts of the hydroxyamino acids serine (3.3 to 2.73%), threonine (1.4 to 1.19%), and hydroxylysine according to Cohn and Edsall ('43). These amino acids contain adjacent hydroxyl and amino groups and should give the Malaprade reaction, and it is recorded that they are oxidized by periodic acid. Reticulin does not appear to have been studied extensively by the protein chemists. It is known to reduce ammine-silver complexes more actively than collagen and to accept selectively sulfonated triphenylmethane dyes from highly acid solutions perhaps more actively than does collagen (Lillie, '45). Serum albumen and globulin apparently contain a variable amount of polysaccharide, and albumen contains 0.6% serine (Hawk, Oser and Summerson, '47).

According to Lison ('36) the chromaffin reaction of adrenal medulla is due to a bichromate oxidation product of adrenalin. With adjacent hydroxyl and methyl amino groups adrenalin itself (3,4 (HO)<sub>2</sub>C<sub>6</sub>H<sub>3</sub>-C(HOH)-CH<sub>2</sub>-NH-CH<sub>3</sub>) could well give the Malaprade reaction, and one of its presumed forestages, 3,4 dihydroxyphenylserine (Schmidt, '44) should give the periodic acid Schiff reaction. Since potassium bichromate in simple aqueous solution fails to produce Schiff reacting alde-

hydes from glycogen, starch, cellulose, mucins, cartilage, etc., as well as chromaffin, and since periodic acid still acts on chromaffin after fixation in unacidified potassium bichromate mixtures, while both the chromaffin reaction and the periodic acid Schiff reaction of the adrenal medulla cells are abolished or much weakened by fixation in Tellyesniczky's 5% acetic, 2.5% potassium bichromate and chromic acid is known to oxidize the hexose polysaccharides and mucopolysaccharides to Schiff reacting substances, it seems indicated that the oxidation by  $\text{K}_2\text{Cr}_2\text{O}_7$  to produce the chromaffin reaction must react on the catechol grouping and not on the hydroxyl methylamino grouping of adrenalin. Catechol gives an immediate dark brown coloration and precipitation in bichromate and bichromate formalin solutions, as well as reducing silver amino hydroxide and periodic acid.

That adrenal "lipofuscin" and intestinal "melanin" which give the ferric ferri cyanide reduction test for "lipofuscin," should also reduce periodic acid and yield an aldehyde grouping is not greatly surprising. However, I know of no "mucopolysaccharide" which gives this "lipofuscin" reaction. The persistence of these pigments after Carnoy's acetic alcohol chloroform fixation, and the lack of appreciable staining of paraffin sections by oil soluble dyes: a negative Ciaccio reaction: would seem to argue against a "lipo"-fuscin nature. "Fuscin" remains applicable as descriptive of their color.

The adrenal "fuscin" pigment seems to be distinct from any of the steroid hormones, in that the latter are oil soluble. None of the ketosteroids formulated in Hawk, Oser and Summerson ('47) reveal a 1,2 glycol or hydroxylamino grouping, but several do present an  $\alpha$ -ketol ( $-\text{CO}-\text{CH}_2\text{OH}$ ) grouping which will reduce ammoniacal silver solutions, and at least some of them form glucuronates in which the necessary glycol configuration does exist.

Accordingly it is suggested that the original interpretation of the Malaprade reaction be returned to, as indicating the

presence of the -CHOH-CHOH-, the -CHOH-CHNH<sub>2</sub>-, or the -CHOH-CHNHR- grouping, without inference as to mucopolysaccharide structure.

Jeanloz ('50) even questions the validity of the periodic acid Schiff reaction as denoting *adjacent* hydroxyls in polysaccharides, noting that "numerous sugars having such groups, such as cellobiose, methyl  $\alpha$ -D-glucopyranoside, methyl n-acetyl- $\alpha$ -D glucosaminide, give a negative reaction." Some strongly reacting substances such as hyaluronic acid and chitin consume only 0.1-0.4 mole for each repeating unit, while starch, glycogen and cellulose consume one for each pair of adjacent free hydroxyls.

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# A HISTOCHEMICAL STUDY OF THE AUTONOMIC GANGLIA OF THE CAT FOLLOWING PROLONGED PREGANGLIONIC STIMULATION <sup>1</sup>

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SIXTEEN FIGURES

## INTRODUCTION

Although a great deal has been written concerning the cytology and cytochemistry of nerve cells in normal and in experimental states, most of the observations, especially those concerned with cytochemistry, have been based on the experimental procedure of nerve fiber degeneration. There have been but few reports relative to the effects on ganglionic cells of prolonged preganglionic stimulation.

In a series of studies of the cytologic changes in nerve cells induced by physiologic stimulation and depression, under experimental conditions, Dolley ('09-'17) observed alterations in the quantity and the distribution of the chromidial substance and in the nucleocytoplasmic ratio. The sequence of these alterations in the Purkinje cells in experimental animals subjected to continued stimulation was described in 1911. According to Dolley's account, the Purkinje cell, which in the resting condition contains a variable amount of extranuclear chromidial substance and little intranuclear chromatin except in the nucleolus, responds to stimulation first by increased production of chromidial substance and

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then becomes progressively hyperchromatic until the initial enlargement of the whole cell reaches its maximum. Following the stage of hyperchromatism, the cell begins to shrink and the hyperchromatism recedes until the chromidial content of the cytoplasm has become reduced to the average normal level, but the nucleus shows evidence of edema. The nucleocytoplasmic ratio, consequently, is shifted in favor of the nucleus. The chromidial content of the cytoplasm suffers still further diminution until the secondary restoration of the cytoplasmic chromidial substance sets in. The newly formed chromidial substance is first piled up about the nuclear membrane and then displaced toward the periphery. Following this stage, secondary diminution of the chromidial substance sets in and continues until no chromatin material remains apparent except in the nucleolus. Finally this also passes out and the cell appears pale and exhausted. During the final stage of exhaustive activity, the nucleocytoplasmic ratio is shifted in favor of the plasma. In view of this succession of changes in the cell, it may be assumed that the chromidial substance in the cytoplasm is used up during cellular activity and that it is continually replaced by the mediation of the nucleus. With long-continued activity the production of chromidial substance no longer equals its consumption and the supply undergoes a progressive diminution until finally no chromidial substance remains in the cell.

In the light of our present knowledge of the chemical composition of the chromidial substance, the findings of Dolley require re-interpretation. Caspersson ('40) and Landström, Caspersson and Wohlfart ('41) have shown that the Nissl bodies consist primarily of nucleoproteins. The nucleotide components probably are of the ribose type and similar to those found in yeast nucleic acid. The basophilic material of the nucleolus has a similar chemical composition.

According to Hydén ('43), the production of cytoplasmic proteins is induced by the nuclear-membrane-nucleolar apparatus. This point of view conforms more closely to the more recent histochemical findings than that of Dolley, ac-



cording to which chromatin and chromidial substance are regarded as essentially similar. In other respects Hydén's findings corroborate those of Dolley.

Comparable studies of the effects of stimulation and depression on autonomic ganglion cells are not available. On the basis of a study of the effects of stimulation of the abdominal viscera on the celiac ganglion cells in the albino rat, Ingersoll ('34) reported a sequence of changes in the chromidial substance and the nucleocytoplasmic ratio. According to his findings, the initial response of the celiac ganglion cells to stimulation appears to be increased production of chromidial substance. This is followed by a decrease in the chromidial substance, a shift of the nucleocytoplasmic ratio in favor of the nucleus, and finally depletion of chromidial substance.

In autonomic ganglia removed surgically in the treatment of patients with peripheral vascular disease, arthritis, etc., as reported by Kuntz ('34), the alterations observed in the ganglion cells resemble those which have been induced in animals by direct and reflex stimulation. They probably represent in a large measure the effects on the ganglion cells of reflex stimulation due to afferent impulses which arise at sites of the vascular lesions. Since such reflex stimulation results in increased vascular tonus at the sites of the lesions, which aggravates the pathologic state and tends to increase the flow of afferent impulses, the ganglion cells are maintained in a state of hyperactivity. These changes include neuronophagia of some of the ganglion cells, diminution of the chromidial substance in the cytoplasm of a large percentage of the ganglion cells, deposition of melanotic pigment in a variable percentage, marked edema of a small number and, in some cases, hyaline degeneration and vacuolization of ganglion cells.

Kuntz and Sulkin ('47a and b) reported hyperplasia of the interstitial tissue in autonomic ganglia including the capsule cells, in experimental animals, induced by prolonged faradic stimulation of preganglionic fibers. Hyperplasia of

the interstitial tissue in autonomic ganglia is also a common phenomenon associated with various pathologic states in man. The cells in question, which appear to be homologous to the oligodendroglia in the central nervous system, undergo amitotic division.

According to Sulkin and Kuntz ('48), preganglionic stimulation of the superior cervical sympathetic ganglion in the cat resulted in changes in the Golgi apparatus in the ganglion cells. These changes consist either of a breakdown of the Golgi network to form discrete Golgi bodies or of complete disappearance of the Golgi apparatus. In preparations of autonomic ganglia of rats with experimental hypertension and of autonomic ganglia of experimental rabbits with hypercholesteremia and marked atherosclerosis, most of the ganglion cells appeared to be devoid of Golgi substance, whereas preparations of ganglia of control animals exhibited a very heavy Golgi network in all the ganglion cells. In another study, these authors ('48) demonstrated the presence of ascorbic acid in both the ganglion cells and the neuroglial supporting cells in autonomic ganglia of the cat, the guinea pig, the rabbit and man. In the cat, ganglion cells which had been subjected to prolonged preganglionic stimulation exhibited a marked decrease in the ascorbic acid content.

The present study has been undertaken to obtain further data relative to the histochemical structure of autonomic ganglion cells. It is based on normal ganglia and ganglia which have been subjected to prolonged preganglionic stimulation.

#### MATERIALS AND METHODS

Sixty adult cats weighing  $3\frac{1}{2}$  to 5 pounds have been used in this investigation. With the animal under nembutal anesthesia, the left superior cervical ganglion was removed and prepared to be used as a control. In many instances the left nodose ganglion which is attached to the superior cervical ganglion was removed together with it. The right cervical sympathetic trunk was then stimulated, by means of a thyro-

tron tube stimulator at 2.5 volts and at a frequency of 10–30 shocks per second, continuously for 8 hours or intermittently for 8 hours a day for two or three days. The right superior cervical ganglion together with the right nodose ganglion were then removed and fixed. Sections of the left control ganglia were mounted on the same slides with sections of the right experimental ganglia and were stained or incubated together.

Alkaline phosphatase was demonstrated by utilizing sodium glycerophosphate, in 5  $\mu$  sections incubated 8 and 24 hours at pH 9.0 following fixation in cold 85% alcohol. This procedure was carried out according to Kabat and Furth's ('41) modification of Gomori's ('39) technique.

For the visualization of acid phosphatase, the method of Gomori ('41) as modified by Wolf, Kabat and Newman ('43) was used. As reported previously (Sulkin and Kuntz, '47), the most consistent results were obtained by using a substrate containing at least 95% sodium beta glycerophosphate, in which the sections were incubated for 24 and 48 hours.

Lillie ('47) has shown that, as McManus ('46) had reported, periodic acid oxidation followed by treatment with the Schiff reagent stains not only glycogen but also mucus, collagenous fibers, basement membranes and a variety of other substances. Glycogen was differentiated from mucus and other substances by incubation of control sections with a buffered solution of 1% malt diastase for one hour at 37°C. This method of digestion is more satisfactory than the older method in which the digestive agent is saliva. Sections, which had been impregnated with collodion on slides, were treated with 1% periodic acid for 10 minutes, stained in the Schiff reagent for 15 minutes and subsequently rinsed in sulphurous acid.

For the demonstration of unsaturated triglycerides, frozen sections fixed in Baker's ('44) formol calcium and imbedded in gelatin were stained with a saturated solution of Nile blue sulphate. A red reaction indicates the presence of unsaturated triglycerides. The blue reaction is non-specific.

Lipoids were demonstrated by using the formol-sudan-black technique described by Baker ('44). Plasmals were studied by treating frozen sections hydrolized with mercuric chloride with the Schiff reagent.

For the study of metachromasia, ganglia were fixed in a 4% aqueous solution of lead subacetate and the deparaffinized sections were stained in a 0.25% or 0.5% aqueous solution of toluidin blue according to the method of Holmgren ('40). Observation of the metachromatic reaction was facilitated by placing a strong yellow filter between the light source and the microscope.

For the study of mitochondria, the ganglia were fixed in Regaud's fixative or by the Kolatchew-Nassonow method and then stained by the technique of Fain and Wolf ('44) or with iron-alum haematoxylin. The best results were obtained by use of the Kolatchew-Nassonow method, followed by complete bleaching of the osmic acid coloration and subsequent staining with iron-alum haematoxylin.

#### OBSERVATIONS

*Alkaline phosphatase.* The cytoplasm of the control autonomic ganglion cells shows a positive alkaline phosphatase activity. But little variability was observed in the cells of the same ganglion or in the ganglia of different animals (fig. 1). The activity within the neuroglial elements is greater than that in the cytoplasm of the ganglion cells. Marked activity occurs also in the nucleoli of the ganglion cells (fig. 3).

Following prolonged preganglionic stimulation, the alkaline phosphatase activity in the cytoplasm of the ganglion cells is greatly diminished but that in the neuroglial elements is increased (fig. 2). This is coincident with a loss of activity in the nucleoli of the ganglion cells (fig. 4). The photomicrographs in figures 1-4 have been taken from sections mounted on the same slide.

*Acid phosphatase.* The intensity of acid phosphatase activity varies considerably in different animals in the series. This is not due to faulty technic, as can be demonstrated in



figures 5 and 6. The photomicrograph in figure 5 was taken from a section of a control superior cervical ganglion which showed very weak activity. The one in figure 6 was taken from a section of the nodose ganglion which was attached to the superior cervical ganglion and showed marked activity. The sensory ganglia of different animals in the series showed very little variation in acid phosphatase activity. It was intense in all the sensory ganglia examined, regardless of the activity observed in the autonomic ganglion cells of the same animal. In a few cats in this series, the acid phosphatase activity in many of the autonomic ganglion cells is as strong as it appears in the sensory ganglia (fig. 7).

Following prolonged stimulation of the preganglionic fibers, the acid phosphatase activity in the ganglion cells is reduced. This is shown in figure 8, which is a photomicrograph taken from a section of a stimulated superior cervical ganglion for which figure 7 represents the control. The photomicrograph in figure 9 was taken from a section of the nodose ganglion which was attached to the stimulated superior cervical ganglion. Unless the acid phosphatase activity in the control ganglion is very slight, prolonged preganglionic stimulation usually is followed by a reduction in this activity.

*Periodic acid.* Sections of autonomic ganglia treated with periodic acid and stained with the Schiff reagent show a variety of staining reactions in the ganglion cells. The amount of periodic acid positive material varies in different cells of the same ganglion as well as in ganglia of different animals. Most of the cells show a positive reaction and in some of them the reaction is of high intensity (fig. 10). The number of cells showing reactions of high intensity varies in the different ganglia. Following digestion with a 1% buffered solution of malt diastase, the reaction completely disappears from the ganglion cells, indicating that the positive reaction demonstrated the presence of glycogen. The neuroglial elements, the nerve fibers and the connective tissues also show a positive reaction which appears uniform, but which is resistant

to the enzymatic digestion. It probably is due to the presence of mucopolysaccharides and phospholipids.

Following prolonged preganglionic stimulation, there is a sharp increase in the number of ganglion cells which shows a positive reaction as well as an increase in its intensity (fig. 11). Under these conditions also the cells are negative following enzymatic digestion. An increase in the glycogen content of the ganglion cells following prolonged stimulation was observed consistently in all the animals that were examined. The positive reacting material in the neuroglial cells, nerve fibers and connective tissue, which resists digestion with diastase, remained unaltered following the prolonged stimulation.

*Nile blue sulphate.* In frozen sections of control autonomic ganglia stained with Nile blue sulphate according to the method of Baker for the demonstration of unsaturated glycerides, the ganglion cells in all the ganglia are stained blue. This indicates a negative reaction. Following prolonged preganglionic stimulation, the staining reaction of most of the ganglion cells remains unaltered. The cytoplasm of occasional ganglion cells, however, stains intensely red (fig. 12). In most of the red staining cells, the nuclei show displacement toward the periphery. The cells also appear swollen. This is a positive reaction with respect to unsaturated glycerides. It was observed in varying numbers of cells in the different experimental ganglia.

*Sudanophilia.* Frozen sections of autonomic ganglia stained with sudan black following formol-calcium fixation show a consistent distribution of sudanophilic bodies throughout the cytoplasm of the ganglion cells. The lipid bodies are in the form of granules of varying sizes and small plaques (fig. 13). Following prolonged preganglionic stimulation, the sudanophilic substances remain unaltered in quantity and distribution in most of the ganglion cells. In occasional cells (fig. 14), however, the cytoplasm appears to be highly sudanophilic and the entire cell appears to be undergoing fatty degeneration. Cells of this type have not been observed in control ganglia.

*Mitochondria.* The mitochondria are uniformly distributed in the cytoplasm of the ganglion cells in the control ganglia in the form of small or larger granules (fig. 15). In the neuroglial cells their arrangement is perinuclear with rows of mitochondrial granules radiating out into the cell processes. Following prolonged preganglionic stimulation, the autonomic ganglion cells exhibit reduction in the numbers and in the sizes of the mitochondria and alteration in their arrangement (fig. 16). The neuroglial elements showed a reduction in size of the mitochondria, but alterations in their concentration could not be recognized due to the crowding of the cells.

*Metachromasia.* In sections of the superior cervical ganglia fixed in lead subacetate and stained with toluidin blue, the different elements show different staining reactions. The ganglion cells appear metachromatic, due to the staining reaction of the chromidial substance. The other cytoplasmic constituents remain unstained. The nucleoli of the ganglion cells appear highly metachromatic. When a yellow filter is used to accentuate the appearance of the staining reaction, the neuroglial nuclei remain blue, the chromidial substance in the ganglion cells appears purple and their nucleoli appear red. Prolonged preganglionic stimulation results in no apparent alteration in the staining reaction of any of these elements.

*Plasmalogen reaction.* In frozen sections of autonomic ganglia treated with mercuric chloride and stained with the Schiff reagent, the cytoplasm of the ganglion cells shows a weak but positive "plasmal" reaction while the nuclei are completely negative. The myelin sheaths of the nerve trunks show a reaction which is much stronger than that of the ganglion cells. Prolonged preganglionic stimulation results in no apparent change in this reaction.

#### DISCUSSION

The data obtained in the present investigation indicate that in the cat the autonomic ganglion cells show a consistent

alkaline phosphatase activity, a variable acid phosphatase activity, variable amounts of glycogen, sudanophilic substances in the form of granules and plaques, a uniform distribution of mitochondria, metachromatic staining properties of the chromidial bodies and the nucleoli, and a positive "plasmal" reaction. Following prolonged preganglionic stimulation the alkaline and the acid phosphatase activity in the cytoplasm of the ganglion cells is diminished, their glycogen content is increased, the mitochondria are reduced in number and undergo alterations in arrangement. Some ganglion cells also show alterations in their lipids.

The view of Gomori ('39) that nerve tissue contains no alkaline phosphatase or only minimal traces of it is not supported by the data obtained in the present investigation. Newman et al. ('50) have demonstrated the presence of small amounts of this substance in the nerve cells in the central nervous system, and of larger amounts in the interstitial tissue. The alkaline phosphatase activity in the autonomic ganglia probably plays a significant role in maintaining the balance of nucleoprotein production in the chromidial bodies and the nucleoli. Jeener ('46) has advanced the hypothesis that cytoplasmic nucleoproteins contain two complexes, one of which comprises ribonucleoproteid, and the other of which is characterized by its high alkaline phosphatase content.

The increase in the alkaline phosphatase activity observed in the neuroglial elements following prolonged preganglionic stimulation also appears to be significant. As has been previously reported (Kuntz and Sulkin, '48a and b), prolonged preganglionic stimulation is accompanied by proliferation of the neuroglial cells in the ganglion. An increase in alkaline phosphatase activity in other actively proliferating tissues has also been reported by numerous investigators (Lipman, '36; Moog, '43, '44; Fell and Danielli, '43; Bourne, '43; and Sulkin and Gardner, '48).

The occurrence of acid phosphatase activity in nerve cells was first recognized by histochemical methods by Wolf, Kabat and Newman ('43). In the central nervous system, according



to their account, the number of stained nerve cells varied from area to area. In one human autonomic ganglion, as reported by them, the cytoplasm of the ganglion cells showed a marked reaction. Bodian and Mellors ('44) reported a high degree of consistency of staining in anterior horn cells of the rhesus monkey. They found a markedly increased acid phosphatase activity in regions of the cytoplasm of nerve cells in which chromatolysis had been produced by section of the axons.

The acid phosphatase activity in the autonomic ganglia of the cat was highly variable, although the attached nodose ganglia showed little variability from animal to animal. The inconsistency in the acid phosphatase activity in the autonomic ganglia of the different cats may be accounted for on the basis of different degrees of functional activity of the autonomic nerves. This assumption appears to be supported by the fact that prolonged stimulation is followed by a reduction in acid phosphatase activity in the autonomic ganglion cells.

The autonomic ganglion cells differ from the neurons in the central nervous system in their glycogen content. According to Kleiner ('48), the brain contains little glycogen. One of us (Sulkin) has examined sections of the brains of young rats prepared by the periodic acid technic and has found that the neurons in most parts of the brain are devoid of glycogen. Appreciable amounts of histochemically demonstrable glycogen were observed only in the choroid plexus. The explanation for the increase in glycogen in the autonomic ganglion cells following prolonged stimulation is obscure. A somewhat analogous situation occurs in the adrenal gland, where during active secretion of cortisone there is a marked drop in ascorbic acid and an increase in glycogen. The relationship of these two situations is purely speculative. According to Gersh and Catchpole ('50), certain phospholipids may give a positive periodic acid reaction in addition to the mucoproteins. It is probable, therefore, that the phospholipids in

the nerve trunks are responsible for the positive reaction observed in the present study.

Our data relative to the nature of the Golgi apparatus in nerve cells, in general, support the views of Gatenby and his associates ('49a and b). Close examination of frozen sections treated with sudan black reveals the true Golgi apparatus as spaces to which granules or plaques of sudanophilic substances may or may not be attached. It is believed that the larger sudanophilic bodies are homologous to those which Gatenby refers to as "Ciaccio bodies." The fine granulations which Gatenby observed can also be seen in our preparations. The Golgi apparatus did not stain with sudan black in the manner reported by Baker ('44) and Thomas ('48).

Our data relative to the quantitative changes in mitochondria following prolonged preganglionic stimulation do not corroborate the findings reported by Strongman ('17) who studied the relationships between mitochondria and the discharge of nerve impulses. Using young white mice, he allowed them to swim in water until they were exhausted, which required from one to two hours. He then studied the cerebral cortex, the cerebellum and the spinal cord. According to his findings, especially in the Purkinje cells in the cerebellum, there is a tendency toward an alteration in distribution of the mitochondria in the cytoplasm of the nerve cells of the fatigued animals as contrasted with the more or less uniform distribution in corresponding nerve cells of the control animals, but he found no diminution in the number of mitochondria. On the basis of our findings in comparison with those of Strongman, it appears that the mitochondria in autonomic ganglion cells are more susceptible to change than those in the neurons in the central nervous system, or that continuous faradic stimulation over a period of 8 hours is more effective than the technique of producing muscular fatigue over a period of one or two hours. Hartmann ('48) reported that there is a progressive increase in the number of mitochondria in the ventral horn cells of the rat during the 11 days immediately following section of the sciatic nerve.

In addition to the numerical changes, his data indicate that the mitochondria exhibit qualitative changes that include a slight increase in size.

The metachromatic staining of chromidial bodies in nerve cells has been reported by other investigators (Windle, Rhines and Rankin, '43; Wislocki, Bunting and Dempsey, '47). According to Wislocki et al., the metachromatic staining of the perikaryon of the nerve cell with toluidin blue can be abolished by previous exposure to ribonuclease. This apparently indicates that the dye may become attached to the ribonucleoproteins in a metachromatic form. Since it has been reported that the nucleoli of nerve cells also contain ribonucleoproteins, the same phenomenon probably explains the metachromatic staining in these elements. The reason for the greater degree of metachromasia in the nucleolus is not known.

According to Albert and Leblond ('46), there is a strict parallelism between the histochemical reactions produced by 2,4-Dinitrophenylhydrazine and Feulgen's plasmal reagent. This reaction is believed to identify in tissue sections a group of substances called "plasmalogens" which comprise acetals of fatty aldehydes (stearic, palmitic, etc.) bound with colamine glycerophosphate (Thannhauser and Schmitt, '46). They occur in the adrenal cortex, the corpus luteum, the mammary and the preputial glands and fat cells. They also occur in some tissues which do not give positive sudanophilic reactions, such as kidney, liver, thyroid epithelium, muscle, prostatic and seminal vesicle epithelium and elastic fibrils. Albert and Leblond ('46) have previously described their occurrence in the myelin sheaths of peripheral nerves as well as in the nerve tracts of the central nervous system. There has been no previous report of their occurrence in nerve cells.

Certain precautions must be taken in interpreting the observations made in this study. It is not known whether any of the alterations that were observed following prolonged preganglionic stimulation are due to direct stimulation of the ganglionic cells or, in part at least, to the result of con-

tinuous vasoconstriction brought about by stimulation of the fibers.

Experience with human ganglia has shown that, in individuals who had suffered from certain infectious diseases, hypertension or peripheral vascular diseases, the ganglion cells have undergone degenerative changes in varying degrees. Factors of this nature may account for variations which have been observed even in control ganglia.

#### SUMMARY

The data obtained in the present study afford a partial basis for a histochemical analysis of autonomic ganglia in the normal state and following prolonged preganglionic stimulation. They include findings relative to the distribution of the alkaline and acid phosphatases, periodic acid positive materials, sudanophilic and Nile blue sulphate positive materials, metachromatic substances, mitochondria and "plasmalogens."

The autonomic ganglia in the cat show a consistent alkaline phosphate activity which is very marked in the neuroglial elements and the nucleoli of the ganglion cells and less marked in the cytoplasm of the ganglion cells. Following prolonged preganglionic stimulation of the ganglion the activity is diminished in the cytoplasm and the nucleoli of the ganglion cells, but increased in the neuroglia.

The acid phosphatase activity varied in the different animals in this series. Following preganglionic stimulation it was diminished in the ganglion cells.

Periodic acid positive materials are found in the cytoplasm of the ganglion cells, the nerve trunk, the neuroglial elements and the connective tissue. This material in the cytoplasm of the ganglion cells consists of glycogen, a substance which markedly increases following prolonged preganglionic stimulation. The other periodic acid positive materials probably consist of mucopolysaccharides and phospholipids.

Nile blue sulphate positive materials were not observed in normal ganglion cells. Following prolonged stimulation, varying numbers of cells showed a strong positive reaction.



Sudanophilic substances are distributed in the form of granules and plaques in the control ganglia. In the experimental ganglia, the cytoplasm of occasional cells became highly and diffusely sudanophilic.

The mitochondria of the ganglion cells occur in the form of small and larger granules uniformly distributed in the cytoplasm. Following prolonged preganglionic stimulation they are diminished in size and number and undergo alterations in arrangement.

The chromidial bodies and the nucleoli of the ganglion cells show a metachromatic reaction. The metachromasia is greater in the nucleoli than in the chromidial substance.

The cytoplasm of the ganglion cells and the nerve trunks show a positive "plasmal" reaction. The reaction is less marked in the cytoplasm of the perikaryon than in the nerve trunks. The nucleus is completely negative.

The significance of the data reported has been discussed.

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## PLATE 1

### EXPLANATION OF FIGURES

1 Section of superior cervical ganglion of a cat removed as control and treated for alkaline phosphatase activity.  $10\times$  ocular;  $20\times$  objective.

2 Section of superior cervical ganglion having undergone prolonged pre-ganglionic stimulation taken from the same cat as figure 1 and photographed from the same slide.  $10\times$  ocular;  $20\times$  objective.

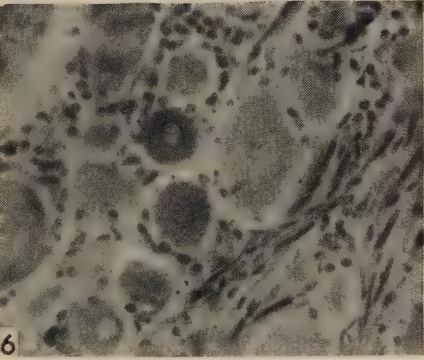
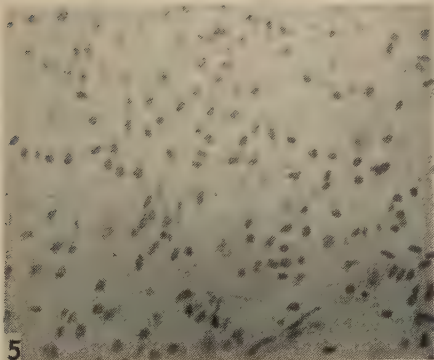
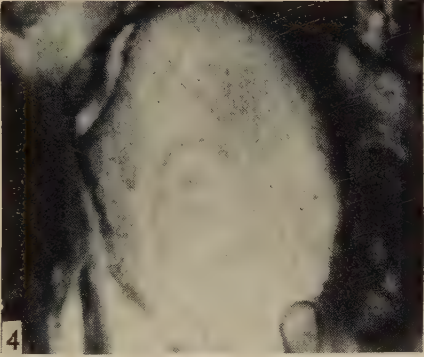
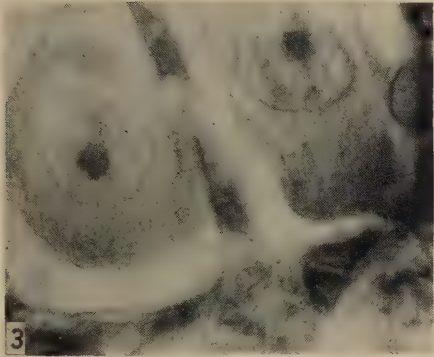
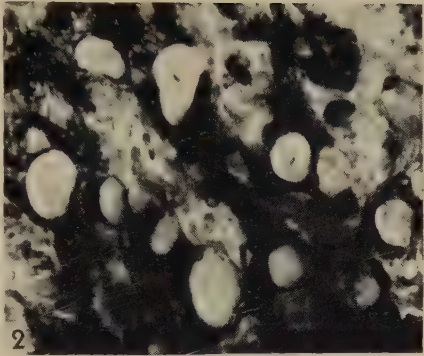
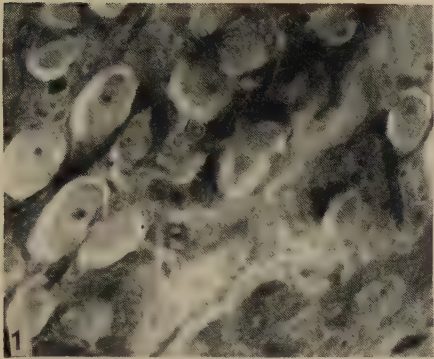
3 Section of superior cervical ganglion of cat treated for alkaline phosphatase activity and photographed at high magnification to show the alkaline phosphatase activity in the nucleolus of the ganglion cell.  $10\times$  ocular;  $90\times$  objective.

4 Section of superior cervical ganglion having undergone prolonged pre-ganglionic stimulation and photographed at high magnification to show loss of alkaline phosphatase activity in the nucleus. This ganglion is the contralateral ganglion to that in figure 3. Both sections were photographed from the same slide.  $10\times$  ocular;  $90\times$  objective.

5 Section of control superior cervical ganglion of cat treated for acid phosphatase activity. This ganglion shows small amounts of activity.  $10\times$  ocular;  $20\times$  objective.

6 Section of nodose ganglion which was attached to superior cervical ganglion in figure 5. Both ganglia were sectioned and stained as a unit. The nodose ganglion shows large amount of acid phosphatase activity.  $10\times$  ocular;  $20\times$  objective.





## PLATE 2

### EXPLANATION OF FIGURES

7 Section of control superior cervical ganglion of cat treated for acid phosphatase activity. This ganglion shows a considerable amount of acid phosphatase activity. 10  $\times$  ocular; 20  $\times$  objective.

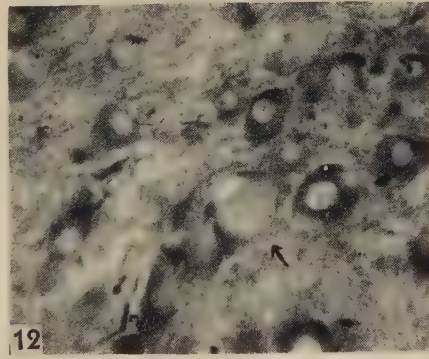
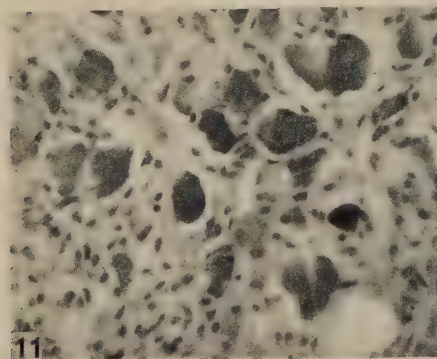
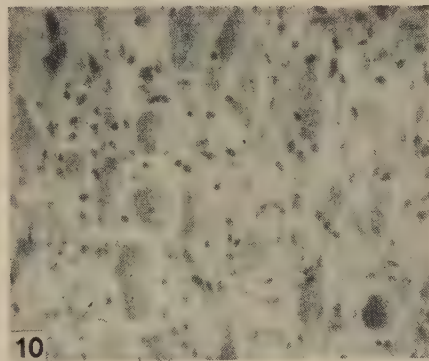
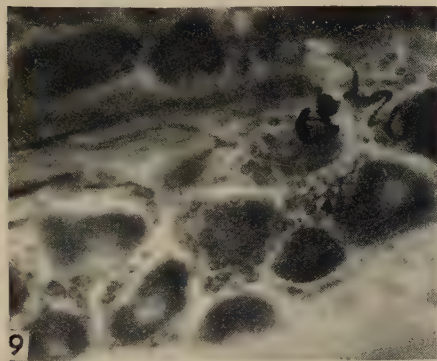
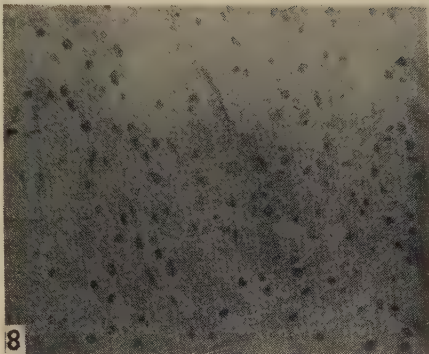
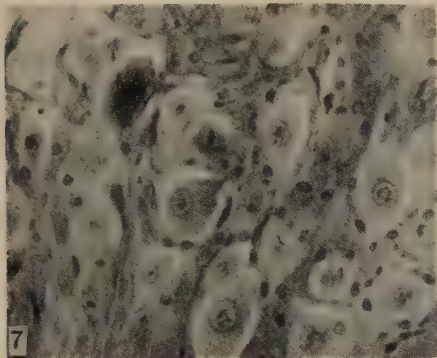
8 Section of superior cervical ganglion of cat having undergone preganglionic stimulation treated for acid phosphatase activity. This is the contralateral ganglion to the one in figure 7. Both ganglia were photographed from the same slide. 10  $\times$  ocular; 20  $\times$  objective.

9 Section of nodose ganglion which was attached to the superior cervical ganglion in figure 7. Both ganglia were sectioned and stained as a unit and were photographed from the same slide. 10  $\times$  ocular; 20  $\times$  objective.

10 Section of superior cervical ganglion of a cat stained with the periodic acid-Schiff reagent. 10  $\times$  ocular; 20  $\times$  objective.

11 Section of contralateral ganglion to that shown in figure 10, which had undergone prolonged preganglionic stimulation and was then stained with the periodic acid-Schiff reagent. Both figures were photographed from the same slide. 10  $\times$  ocular; 20  $\times$  objective.

12 Photograph of a frozen section of the superior cervical ganglion of a cat which had undergone prolonged preganglionic stimulation and was stained with Nile blue sulphate. Arrow points to ganglion cell showing a positive reaction. 10  $\times$  ocular; 20  $\times$  objective.



### PLATE 3

#### EXPLANATION OF FIGURES

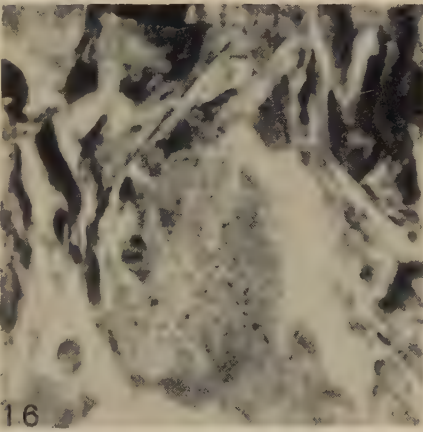
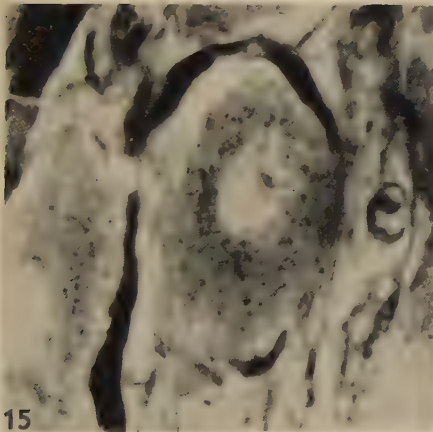
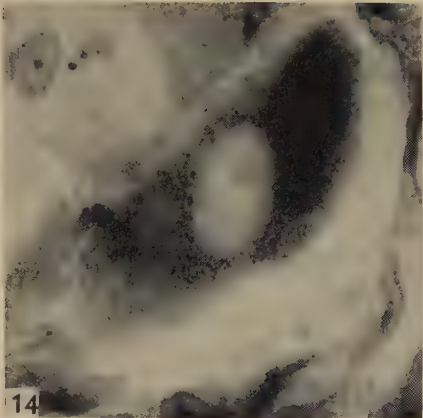
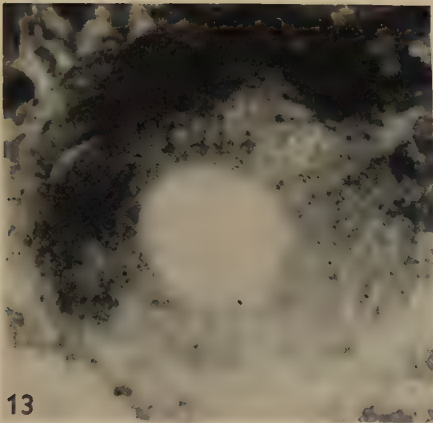
13 Photomicrograph of frozen section of superior cervical ganglion in a cat stained with sudan black. 10  $\times$  ocular; 90  $\times$  objective.

14 Photomicrograph of frozen section of a superior cervical ganglion in the same cat as in figure 13 stained with sudan black. Only occasional cells stain in this diffuse manner. 10  $\times$  ocular; 90  $\times$  objective.

15 Section of superior cervical ganglion of a cat prepared by the Kolatchew-Nassonow method and stained with iron-alum haematoxylin to demonstrate the distribution of mitochondria. 10  $\times$  ocular; 90  $\times$  objective.

16 Section of contralateral ganglion to that in figure 15, which had undergone prolonged preganglionic stimulation and was then prepared in the same manner and photographed from the same slide. 10  $\times$  ocular; 90  $\times$  objective.







# IN VIVO AFFINITY OF DIAMINOACRIDINES FOR NUCLEI <sup>1</sup>

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FIFTEEN FIGURES

## INTRODUCTION

A number of investigations are recorded in the literature reporting that it is possible to stain nuclei in vivo (see reviews, Alexandrov, '33; Becker, '36). Nevertheless, it is generally held that the nucleus of the living cell resists staining and that staining of the nucleus indicates cell damage or even cell death. This is because in most of the studies dealing with the vital staining of nuclei, the cells were stainable only under deleterious conditions. Thus, Nassonov ('30, '32), Alexandrov ('33), Kamnov ('34), Makarov ('34), Gersch ('31), Ries ('37), and others, working particularly with neutral red, produced staining of nuclei in vivo by submitting the cells to such conditions as anoxia, acidosis and hypertonicity. In contrast to this, Ellinger and Hirt, in a series of papers ('29, '30, '31) primarily dealing with the excretion of fluorescein and acriflavine (trypaflavine) in the liver and kidney of the frog and the liver of the rat, reported that the nuclei of the cells of these organs stained in vivo with acriflavine, without any special treatment. Their studies were carried out on living organs in situ by means of vertical illumination.

<sup>1</sup>Aided by grants from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council and the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

This exceptional behavior of acriflavine is of considerable interest in that it opens the possibility of localized irradiation of cell nuclei through the incorporation of tritium (a short range  $\beta$ -emitter) in this or related compounds which accumulate selectively in the nucleus. It is accordingly important to make preliminary inquiry into the universality of the phenomenon of in vivo nuclear staining by acridine compounds. We have paid particular attention to the relative affinity of cells of different tissues and organs for these compounds and to the "vital" character of this phenomenon. The general toxicity of these dyes has also been investigated.

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The authors wish to express their thanks to Dr. Weldon G. Brown, Department of Chemistry, University of Chicago; to Dr. D. L. Tabern, of Abbott Laboratories, and to the American Cyanamid Company, who have generously supplied them with acridine and with acridine related compounds, which could not be obtained commercially.

#### MATERIALS AND METHODS

The following is a list of compounds tested:<sup>2</sup>

Acriflavine hydrochloride (mixture of the hydrochlorides of 3,6-diamino-10-methylacridinium chloride and 3,6-diaminoacridine).

Acriflavine neutral (mixture of 3,6-diamino-10-methylacridium chloride and 3,6-diaminoacridine).

Proflavine (3,6-diaminoacridine-monohydrogen sulfate).

Proflavine hydrochloride (3,6-diaminoacridine hydrochloride).

3,6-diamino-10-methylacridinium chloride.<sup>3</sup>

Acridine orange (3,6-bis(dimethylamino)-acridine).

Acridine yellow (2,7-dimethyl-3,6-diaminoacridine).

Rivanol (6,9-diamino-2-ethoxyacridine lactate).

9-aminoacridine hydrochloride.

<sup>2</sup> The numbering of the acridine ring is in accordance with the system used by Chemical Abstracts.

<sup>3</sup> Prepared from acriflavine hydrochloride according to the method described by Marshall ('34).



Phosphine GNR.<sup>4</sup>

"2-methoxy-6-chloro-9-diethylamino propylaminoacridine."<sup>4</sup>

"9-(10)-acridinethione."<sup>4</sup>

2-methoxy-6-chloro-9-(2-pyridylamino)-acridine.<sup>4</sup>

2-methoxy-6-chloro-9-(2-thiazylamino)-acridine.<sup>4</sup>

3-(p-sulfamylbenzeneazo)-acridine.<sup>4</sup>

2-methoxy-6-chloro-9-cyanamido-acridine.<sup>4</sup>

Flaveosine (Schultz 913).<sup>5</sup>

10-methylacridinium chloride.<sup>5</sup>

N, N'-dimethyldiacridinium dinitrate.<sup>5</sup>

9-chloro-9(p-dimethylaminophenyl)-10-methylacridane.<sup>6</sup>

9-chloro-9-phenyl-2-anilino-10-methylacridane.<sup>6</sup>

9-chloro-9-(p-dimethylaminophenyl)-3-dimethylamino-10-methylacridane.<sup>6</sup>

9-chloro-9(dimethylaminophenyl)-10-phenylacridane.<sup>6</sup>

9-chloro-9(p-hexyloxyphenyl)-10-methylacridane.<sup>6</sup>

The localization of these compounds was studied in tissue sections and observed by virtue of the fluorescence excited by near ultraviolet light. An AH-4 mercury lamp was used as a light source with a 3.4 mm (standard thickness) Corning no. 5113 filter. This filter transmits light from 340 mμ to 500 mμ with a peak at 410 mμ, which is sufficiently close to the absorption maxima of these compounds to produce fluorescence. Since this filter transmits a considerable amount of visible blue, it was necessary to use a yellow no. 3486 filter at the ocular. This eliminates all light below 500 mμ and allows passage of all light above this wave length, including the yellow-green light emitted by these dyes. No quartz is necessary in the optical system at this wave length.

The materials were administered in aqueous solutions in concentrations varying from 0.1 to 1.0%. The dosages varied from 12.5 to 25 mg per kg. Fresh solutions were made for each experiment and were administered either parenterally or orally. The animals were anaesthetized and sacrificed at various intervals after administration as detailed below. Rabbits, guinea pigs, rats and mice were used.

<sup>4</sup> American Cyanamid Company.

<sup>5</sup> Dr. Weldon G. Brown, Department of Chemistry, University of Chicago.

<sup>6</sup> Abbott Laboratories.

The ordinary fixation methods (Zenker-formol, 10% formalin, osmic acid, Bouin, picric acid, 95% alcohol) could not be used, as they either quenched the fluorescence, caused changes in the distribution of the dye, or extracted the dye. However, the fresh-frozen section technique and the freezing-drying technique of Altmann-Gersh were found to be satisfactory.

The fresh-frozen technique has the advantage of being rapid but this material does not keep well and in addition the initially sharp nuclear fluorescence becomes diffuse throughout the cell within 24 hours in these preparations. The experiments with different compounds on the relative staining intensities of nuclei of cells in different tissues and organs were done exclusively with this method. To avoid errors in the evaluation of differences in fluorescent intensity caused by the deterioration of the fluorescence, the sections were photographed immediately after preparation and comparisons were based on these photographs. The photographic procedures were executed under constant conditions with respect to light intensity, exposure and development.

The freezing-drying method reduces to a minimum possible changes in localization of acridine dyes by diffusion. Also the nuclear localization in the mounted sections is more permanent. In addition, the preparations are superior, in general histological aspects, to those prepared with the fresh-frozen technique. Small bits of tissue were frozen in isopentane cooled in liquid nitrogen, and dehydrated in vacuo at  $-40^{\circ}\text{C}$ . The tissues were embedded in paraffin in the original vacuum. In order to remove the paraffin and to flatten the sections, they were floated on a drop of 13% paraffin in xylol on the slide. A coverslip was added, the excess solution removed with blotting paper and the preparation surrounded with paraffin. The nuclear fluorescence began perceptibly to fade in the frozen-dried preparation within a few minutes of exposure to the ultraviolet illumination. This fading is less rapid in the fresh-frozen sections.

The toxicity of the dyes was determined by estimation of the LD<sub>50</sub>/10 days for mice.

## OBSERVATIONS

Of the compounds tested, the following were found to stain nuclei in vivo: acriflavine HCl, acriflavine neutral, proflavine HCl, acridine orange, acridine yellow, phosphine GNR, rivanol (lactate), and 3,6-diamino-10-methylacridinium chloride. All of these are diaminoacridines.

All of the fluorescence described in this paper is caused by the presence of diaminoacridines since it did not occur in material from animals not given these dyes. No mention will be made of naturally occurring fluorescence, which was easily distinguished from the diaminoacridine fluorescence by either the difference in fluorescent color or by its presence in material from animals not given diaminoacridine compounds.

*General cytological aspects*

The nuclear fluorescence appears almost immediately after injection and with most dyes it reaches peak intensity in one to two hours; with phosphine GNR and acridine orange, maximum intensity is reached at 4 hours. Individual differences with respect to the intensity and duration of staining of the nuclei of different tissue elements and organs are reported below.

The fluorescence in nuclei stained in vivo with any of the above mentioned diaminoacridines is discrete rather than diffuse and it resembles the chromatin pattern after staining with ordinary dyes (fig. 3). This resemblance between the nuclear staining in vivo with diaminoacridines and the ordinary chromatin staining in fixed preparations was consistently noted in all tissues that were investigated. The in vivo affinity of diaminoacridines for nuclear material is especially pronounced during mitosis (fig. 4).

In some cases, in addition to the nuclear staining, there is some staining of the cytoplasm. However, the intensity of the nuclear staining exceeds considerably that of the cytoplasm with the single exception of the adrenal medulla (fig. 5). Here the cytoplasmatic fluorescence is so strong as to "overshadow" any nuclear fluorescence which may

have been present. We have no explanation for this exceptional staining in the adrenal medulla. The distribution of the cytoplasmic stain is always diffuse and we have not seen local staining of Golgi apparatus, mitochondria or cellular inclusions with any of the diaminoacridines used.

### *Tissues and organ systems*

Under our experimental conditions, the nuclei of the cells of all tissues and organs stain *in vivo* with great reproducibility with the exception of the central nervous system and the testis. The central nervous system, in general, does not stain at non-toxic dose levels. Only if the amount injected is at dose levels which are immediately lethal, a faint general staining of the nuclei of these cells occurs. However, the hypophysis and its stalk, as well as the area postrema, stain moderately at non-toxic dose levels. The nuclei of the peripheral ganglia can be stained, though only in moderate intensity in comparison, for example, to the nuclei of the connective tissue cells in the ganglion (fig. 6). The protoplasm of the ganglion cells fluoresces faintly. It is difficult to say whether this fluorescence represents a staining of the Nissl substance or not. The exceptional behavior of the testis is of a different nature. In a large series of mice, nuclear fluorescence of the cells of this organ occurred sporadically and apparently independent of the type of diaminoacridine, the dose or the time after the injection (fig. 1). When nuclear staining was achieved, it was present in all cell types in this organ.

It is not our purpose to describe in detail the *in vivo* staining characteristics of the nuclei of all the organs and cell systems of the organism with diaminoacridines. Based primarily on observations with acriflavine HCl, the main features can be described as follows:

1. In a given organ, the nuclei of cells of the same type of tissues stain with the same degree of intensity and for that given organ, the variations in staining intensity occur primarily among cells of different tissues. For example, in



the uterine mucosa, the nuclei of the connective tissue of the endometrium stain in general much stronger than the nuclei of the epithelial cells (fig. 7). In the bladder (fig. 8), there is a striking difference between the bright fluorescence of the nuclei of the connective tissue cells and the dimmer fluorescence of the nuclei of the epithelial cells. In lymphatic tissue (fig. 9), bone marrow and spleen, the nuclei of a great number of reticular cells fluoresce brighter than those of the free blood cells. The epithelial reticular cells of the thymus behave in the same manner. At present it is difficult to say whether this reaction is specific; in other words, whether all the reticular cells stain consistently brighter than the free blood cells.

There are exceptions to the rule that in a given organ, the nuclei of a given tissue stain with the same intensity. In these instances, the nuclei of a given cell type show differences in fluorescent intensity depending on their location. An interesting example can be found in lymphatic tissue. Here, the nuclei of the small lymphocytes of the corona of the nodule stain brighter than the nuclei of the lymphocytes elsewhere (fig. 9). This difference in intensity of staining is not related to cell size since the small lymphocytes in diffuse lymphatic tissue do not have the intensity seen in those of the corona of the nodule. In the stratified squamous epithelium of the mucous membrane of the tongue (fig. 10) and esophagus, the nuclei of the basal cell layer fluoresce decidedly brighter than the nuclei of the other cells of the epithelium. Something similar occurs in the crypts of the small intestine: the nuclei of the basal parts of the crypts fluoresce stronger than those of other parts of the intestinal epithelium.

The kidney presents variations in staining intensity which are related to the excretion of the dye in this organ. In general, it can be said that the nuclei of the glomeruli stain first after the injection and that the nuclear staining proceeds from here into the tubular system. The nuclei of the glomeruli lose their fluorescence earlier than the nuclei of

the tubules (figs. 11 and 12). The medulla retains the nuclear fluorescence longest.

2. From organ to organ, the affinity in vivo of diaminoacridines for nuclei is not specific for the cells of any given tissue. For example, the nuclei of the connective tissue components of different organs behave differently. They fluoresce brightly in the pancreas, moderately in the thyroid, and little or not at all in the parathyroid. The epithelial elements of the colon fluoresce brightly in contrast to the connective tissue cells, while the reverse is true in the uterine mucosa. In the ovary, the nuclei of the germinal epithelium, as well as those of the follicular epithelium, fluoresce at lower intensity than the connective tissue nuclei of the stroma, but even in this tissue there is no uniformity: the nuclei of the theca cells exceed in brilliance those of the stroma cells (fig. 13).

The behavior of the epithelial elements in the pancreas is peculiar. The epithelial nuclei in the islands of Langerhans fluoresce quite brightly while the nuclei of the epithelium cells in the exocrine portion of this organ are only moderately fluorescent (fig. 14). This difference is not a specific indicator of endocrine function of the islands, as can be seen in the parathyroid glands, where the nuclei are only weakly fluorescent in comparison to the brightly fluorescing nuclei of the thyroid (fig. 15).

*Differences in nuclear affinity of various  
diaminoacridines*

As mentioned above, different diaminoacridines have individual differences with respect to the intensity and duration of staining of the nuclei of various cells. This was examined in kidney, spleen, lung, liver, tongue, small intestine, heart and testis by comparing the fluorescence at various intervals after the injection. The evaluation of intensity and duration of the nuclear fluorescence in these organs was judged by the fluorescence of the brightest components. Results are summarized in figure 1. It can be

seen that there are great differences among the various diaminoacridines in the duration of staining. These differences in duration of staining may, in part, be caused by differences in dye content of the commercial product. Nothing was known about the purity of the materials used with the exception of 3,6-diamino-10-methylacridinium chloride, which was of a high degree of purity<sup>7</sup> and also stained for the longest time. It is improbable, however, that purity alone

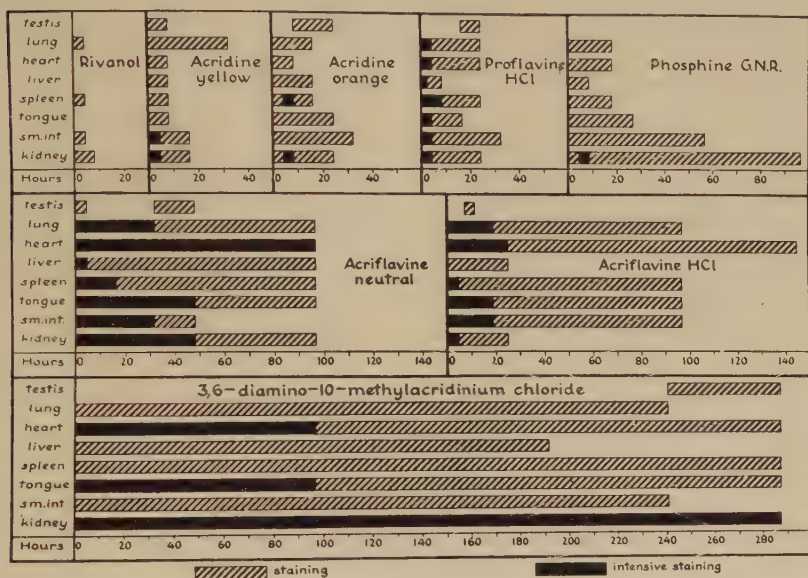


Fig. 1 Duration of nuclear staining of 8 diaminoacridines in 8 different organs.

can explain such extreme differences in duration of staining as occur, for example, between 3,6-diamino-10-methylacridinium chloride and phosphine GNR.

In addition, figure 1 demonstrates that a certain organ specificity of the different compounds is present, since the

<sup>7</sup>The elementary analysis done by Mr. H. Heller in the Department of Chemistry of the University of Chicago gave:  $C_{80.2}H_{5.6}N_{15.0}Cl_{12.7}$ . This is in good agreement with the values calculated for 3,6-diamino-10-methylacridinium chloride with one molecule of water of crystallization.

differences in duration of nuclear staining of different organs do not vary proportionally from dye to dye. This can be seen most strikingly in the heart and the kidney stained with phosphine and acriflavine HCl. The kidney retains phosphine longer than any of the other organs tested, while the heart is stained shorter than the other organs. The reverse is true with acriflavine HCl. Several similar examples illustrating this point can be found in the graphic representation of figure 1. The experiments summarized in this graph also illustrate the erratic behavior of the testis.

TABLE 1  
*Subcutaneous toxicity of diaminoacridines for mice*

| DYE                                           | APPROX.<br>LD50/10 DAYS<br>IN<br>MG/KG | NUMBER<br>OF<br>ANIMALS <sup>1</sup> | MAX. DOSE<br>FOR 0%<br>MORTALITY<br>IN MG/KG | MIN. DOSE<br>FOR 100%<br>MORTALITY<br>IN MG/KG |
|-----------------------------------------------|----------------------------------------|--------------------------------------|----------------------------------------------|------------------------------------------------|
| Acridine orange                               | 630                                    | 39                                   | 300                                          | 900                                            |
| Proflavine HCl                                | 368                                    | 18                                   | 350                                          | 400                                            |
| Rivanol                                       | 225                                    | 51                                   | 100                                          | 325                                            |
| Acridine yellow                               | 195                                    | 108                                  | 50                                           | 350                                            |
| Phosphine GNR                                 | 108                                    | 12                                   | 50                                           | 175                                            |
| Acriflavine HCl                               | 92                                     | 12                                   | 85                                           | 100                                            |
| Acriflavine neutral                           | 45                                     | 9                                    | 40                                           | 50                                             |
| 3,6-diamino-10-methyl-<br>acridinium chloride | 26                                     | 15                                   | 15                                           | 35                                             |

<sup>1</sup> Excluding animals from the groups having 0% and 100% mortality.

### *Toxicity*

The LD50 data (table 1) indicate that in general the longer staining compounds are the most toxic, although there is not a complete correlation between duration of staining and toxicity, in all instances.

It must be stressed that the *in vivo* staining of the nuclei takes place at dose levels significantly below the LD50 quantity.



*Vitality of the cells with intranuclear diaminoacridines*

The question of the vitality of the cells in which the nuclei are stained at non-toxic dose levels is of importance. The fact that all the nuclei in most organs take up dye with no apparent acute ill-effect on the animal indicates that any damage the cells may suffer as a result of the staining cannot be of immediate vital significance. We have examined this further in tissue cultures. A series of hanging drop tissue cultures was made of the buffy coat of the blood of rabbits which were stained in vivo with acriflavine hydrochloride. These tissue cultures did not behave in any way differently from cultures of blood cells of non-injected animals, with respect to the migration of the leucocytes and the formation of macrophages.

In view of the apparent lack of cellular damage as a result of in vivo nuclear staining with diaminoacridines, it seemed of interest to investigate any effect they may have on growth dependent on cell division. The regeneration of the rat liver after partial removal was chosen as a test object because its rapid growth after the first day is mainly due to cell division and because the degree of regeneration at a given time can be easily measured. Hepatectomies and calculations of the degree of regeneration were done according to the methods of Brues et al. ('36). Eight rats between 100–200 gm body weight were hepatectomized and injected with acriflavine HCl. One rat was killed on the second and the 6th day and two on each intervening day. Each rat was given a daily subcutaneous injection of a sublethal staining dose of acriflavine hydrochloride (125 mg per kilogram) with the exception that the animals killed on the 4th and 5th days did not receive injections on these days. Simultaneously, 10 rats from the same stock were hepatectomized and killed in pairs daily from the second to the 6th day. These served as controls. Calculations of the percent regeneration in these two groups are shown graphically in figure 2. The period of most rapid cell division occurs dur-

ing the second and third day, where the curves are practically identical. The difference between the two curves on the 4th and 5th day is within the normal variation of regeneration at any given time. These data show that there is no significant depression in the rate of growth in the livers, the

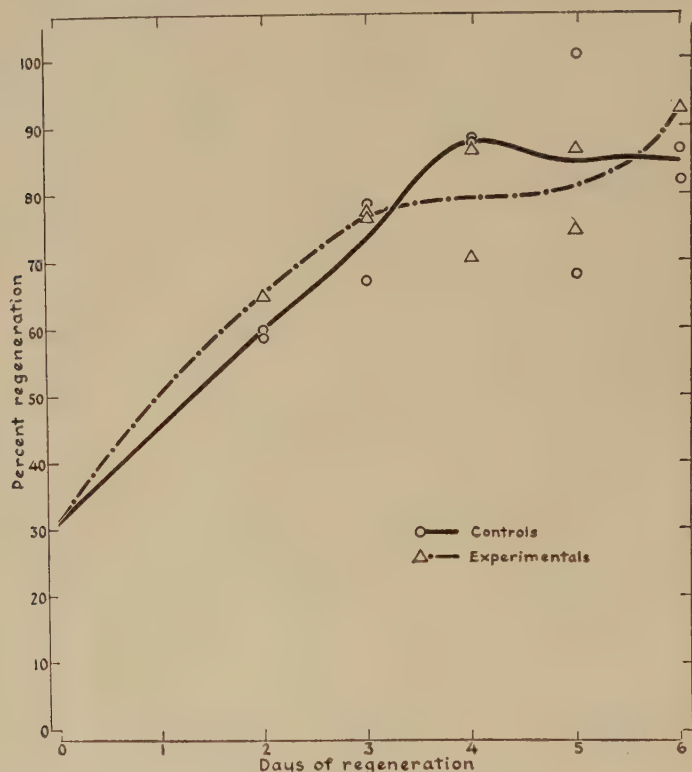


Fig. 2 Regeneration of rat livers after partial removal, with and without repeated administration of acriflavine HCl (see text).

nuclei of which contain acriflavine HCl as compared to the livers free of this material. Sections of this material revealed a great number of abnormal mitosis (scattering, clumping and lagging of chromosomes), in both the acriflavine HCl livers as well as in the regenerating livers of the control animals.

## DISCUSSION

It can be stated conclusively that the *in vivo* affinity of diaminoacridines for nuclei does not depend on any experimental intervention in normal cell life, such as anoxia, acidosis, hypertonicity, etc. Conversely, the present evidence indicates that the *in vivo* staining of nuclei with diaminoacridines is possible at dose levels which do not affect the vitality of the cell.

A number of toxicity studies with diaminoacridines (particularly acriflavine) have been done with reference to their bactericidal properties. The investigations of Albert, Francis, Garrod and Linnel ('38) and of Browning ('43) have shown that the toxicity of acriflavine for leucocytes *in vitro* is low and not present at dose levels which have a bactericidal effect. Blacklock ('29) found that the local application of diaminoacridines does not prevent the occurrence of mitoses in regenerating wound surfaces. Mueller ('19), comparing the toxicity of acriflavine for tissues and bacteria, found that connective tissue growth continued in cultures of embryonic chicken tissues in the presence of 0.025 mg acriflavine per 1 ml medium. On the other hand, Bucher ('39) reports that in cultures of rabbit fibroblasts, trypanflavine (acriflavine), in a concentration as low as 1:600,000, completely prevents the occurrence of mitosis. His observations are in agreement with those of Dustin ('29), who classifies trypanflavine (acriflavine) with a number of substances, which he designates as "poisons caryoclasiques," because of their damaging effect *in vivo* primarily on the nucleus and their inhibitive action on mitosis. These observations on the toxic effect of diaminoacridines were not made with reference to the nuclear staining *in vivo* and for this reason they are not essentially relevant to the problem at hand. We have been primarily concerned as to whether nuclear staining *in vivo* with diaminoacridines is necessarily accompanied by a toxic action. The present evidence indicates that this is not so and in addition that mitotic regeneration of cells with nuclei stained *in vivo* proceeds unhampered.

The intensity of nuclear staining varies in different tissues and organs, but is predictable in the sense that the relative intensity in different cell types and tissues is comparable from animal to animal. This is not true for the testis. It seems reasonable to assume that in this organ specific biological factors inherent in the testis rather than technical procedures play a role in the erratic staining of the nuclei of the cells of this organ.

The nuclei of the cells of the central nervous system consistently do not stain with diaminoacridines *in vivo*. The nuclei of the cells of the hypophysis, its stalk and the area postrema, take up the dyes. These areas, and in addition part of the tuber cinereum, the epiphysis, the paraphysis, the wall of the optic recess, and the supraoptic crest, have been reported to stain *in vivo* with trypanblue (Wislocki and King, '30). We have not investigated these other areas. The similarity is remarkable, particularly in view of the fact that Wislocki and King obtained their results with acid dyes, which localized in the cytoplasm, while in the present work basic dyes are used, localizing in the nuclei.

We have not been able to find an explanation of general applicability for the variations in intensity of the *in vivo* staining with diaminoacridines of nuclei of the different organs and cell systems. A number of factors have been considered. The vascular supply, in general, cannot be of great importance. Nuclei of different cell types located in the same tissue often show striking differences in fluorescence intensity, as for example, the reticular cells and the free blood cells in the hemapoietic organs. Another example is the variation in intensity in the endocrine organs, all of which have an intense vascular supply.

Factors such as membrane permeability and nucleoprotein concentration may play a role in the variations of fluorescent intensity of different nuclei; however, at present, it is very difficult to speculate with any probability of accuracy concerning the extent to which they are involved.



There are certain general chemical factors which are known to influence the intensity of fluorescence and which may influence the intensity of fluorescence of nuclei stained *in vivo* with the diaminoacridines. Variations in the hydrogen ion concentration can cause changes in intensity of fluorescence of a number of substances. Of the diaminoacridines used in the present work, acridine orange has been reported by Jensen ('33) to increase its fluorescent intensity over a range of pH 8.4–pH 10.4. Such pH values are not expected to occur in the living nucleus. More important is perhaps the quenching of fluorescence by neutral salts or organic compounds. It is conceivable that the variation in fluorescent intensity in nuclei of different cells is caused by quenching substances present in different concentrations. It must be emphasized here that the variations in fluorescent intensity of the various types of nuclei do not necessarily indicate proportional differences in concentration of the diaminoacridines present. We draw attention to the fact that the fluorescence intensity/concentration curves of a great number of fluorescent substances show very pronounced maxima, and proportionality is present only over a narrow range.

#### SUMMARY

The nuclei of the cells of all tissues and organs, excepting those of the cells of the central nervous system, can be stained in the living organism by parenteral or oral administration of diaminoacridines. The *in vivo* staining of nuclei with these compounds can be observed by virtue of the visible fluorescence of these compounds excited by near ultraviolet light. The intranuclear localization of diaminoacridines resembles the chromatin pattern as revealed by the ordinary nuclear dyes. The intensity of the nuclear fluorescence varies in different tissues and organs. In a given organ, the nuclei of cells of the same type of tissue stain with the same degree of intensity and for that given organ, the variations in staining intensity occur primarily among cells of different tissues. However, from organ to organ,

the affinity in vivo of diaminoacridines for nuclei is not specific for the cells of any given tissue.

The nuclear staining in vivo with these compounds neither causes nor results from any perceptible toxic or degenerative changes. Tissue cultures of leucocytes of animals stained in vivo with diaminoacridine do not behave differently from cultures of animals, which have not received this material. The regeneration of the partially hepatectomized rat liver continues in the presence of intranuclear diaminoacridines and proceeds at the same rate as in animals not injected with these compounds.

Possible causative factors of the observed variations in fluorescent intensity are discussed.

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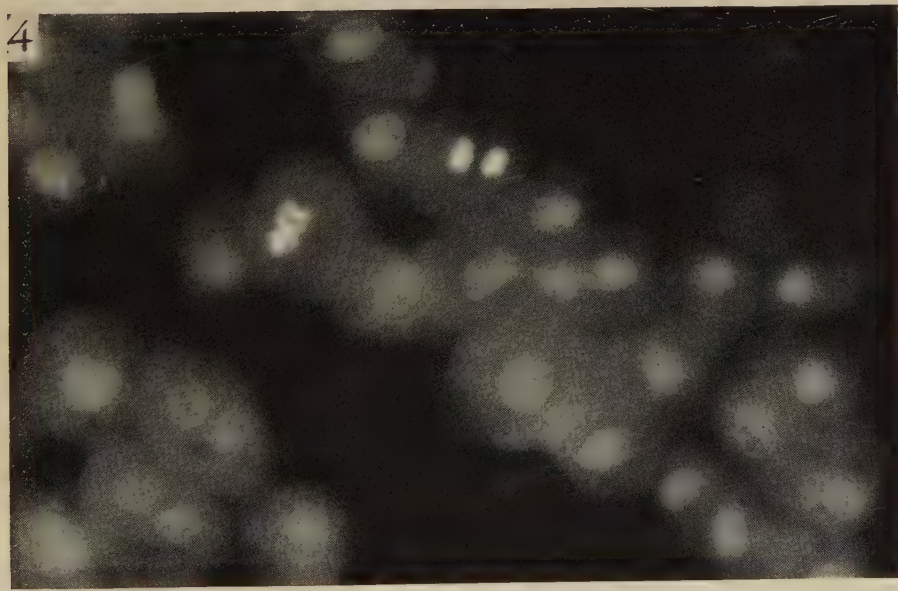
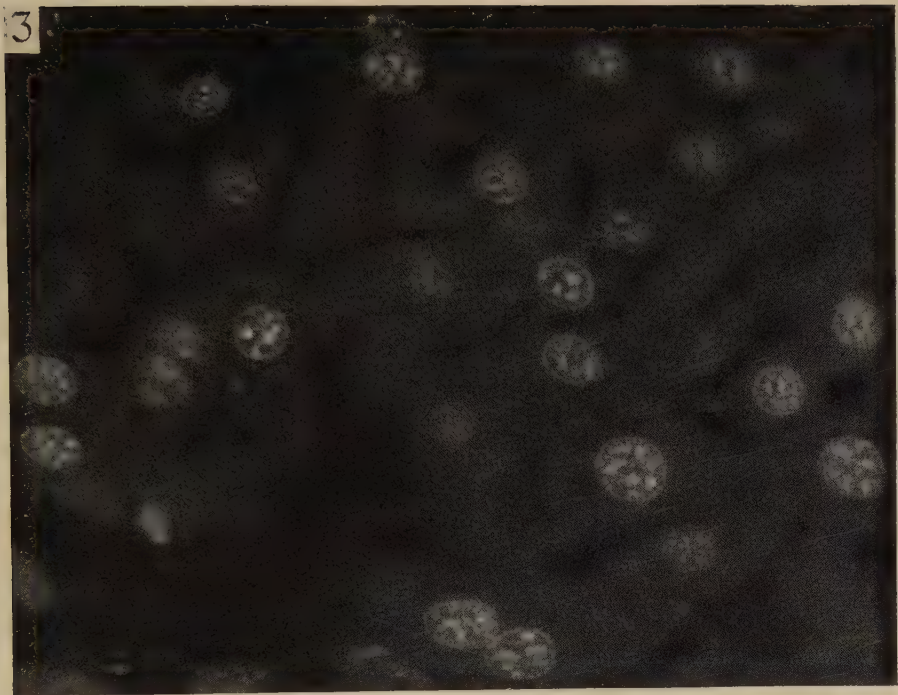
## PLATE 1

### EXPLANATION OF FIGURES

3 Liver, mouse. Acriflavine HCl, 12.5 mg per kilogram, intravenous. Two hours after injection. Frozen-dried.  $\times 600$ .

4 Regenerating liver, rat. Two days after partial hepatectomy. Acriflavine HCl, 12.5 mg per kilogram, intraperitoneal. Thirty minutes after injection. The chromatin material of the dividing nuclei fluoresces stronger than the nuclei of the resting cells. Smear.  $\times 600$ .



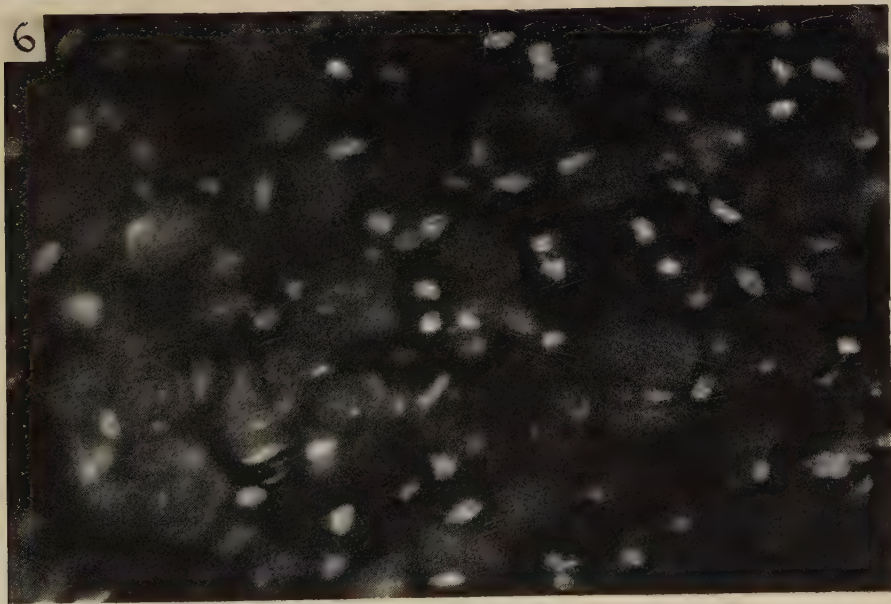
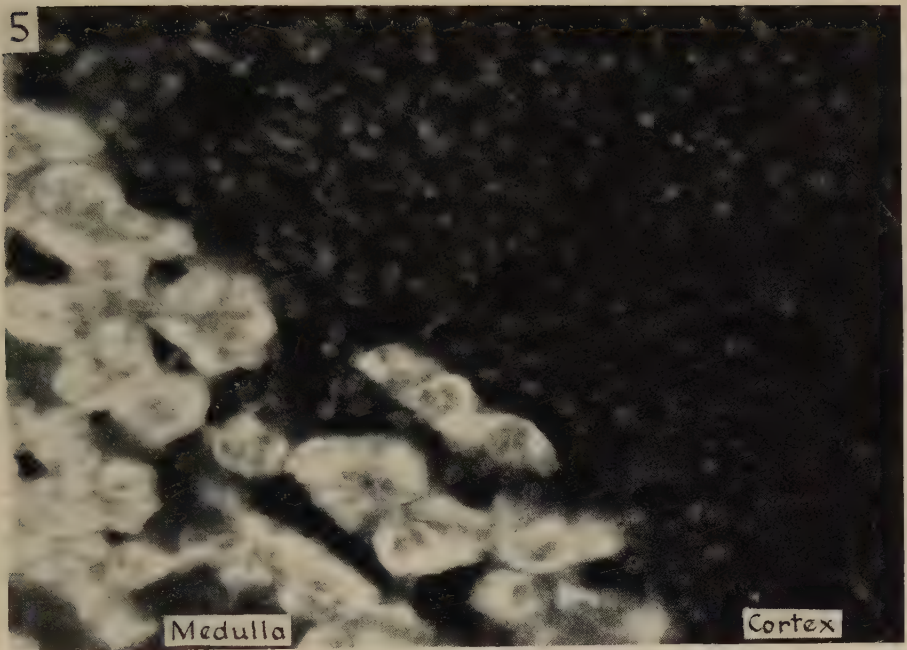


## PLATE 2

### EXPLANATION OF FIGURES

5 Adrenal gland, rabbit. Acriflavine HCl, 12.5 per kilogram, intravenous. Two hours after injection. Note the bright cytoplasmic fluorescence of medulla. Frozen-dried.  $\times 300$ .

6 Sympathetic ganglion, mouse. Acriflavine HCl, 12.5 mg per kilogram, intravenous. One hour after injection. Frozen-dried.  $\times 600$ .



### PLATE 3

#### EXPLANATION OF FIGURES

- 7 Uterus mucosa, rabbit. Acriflavine HCl, 12.5 mg per kilogram, intravenous. One and one-half hours after injection. Frozen-dried.  $\times 600$ .
- 8 Bladder, guinea pig. Acriflavine HCl, 12.5 mg per kilogram, intraperitoneal. Two hours after injection. Frozen-dried.  $\times 300$ .



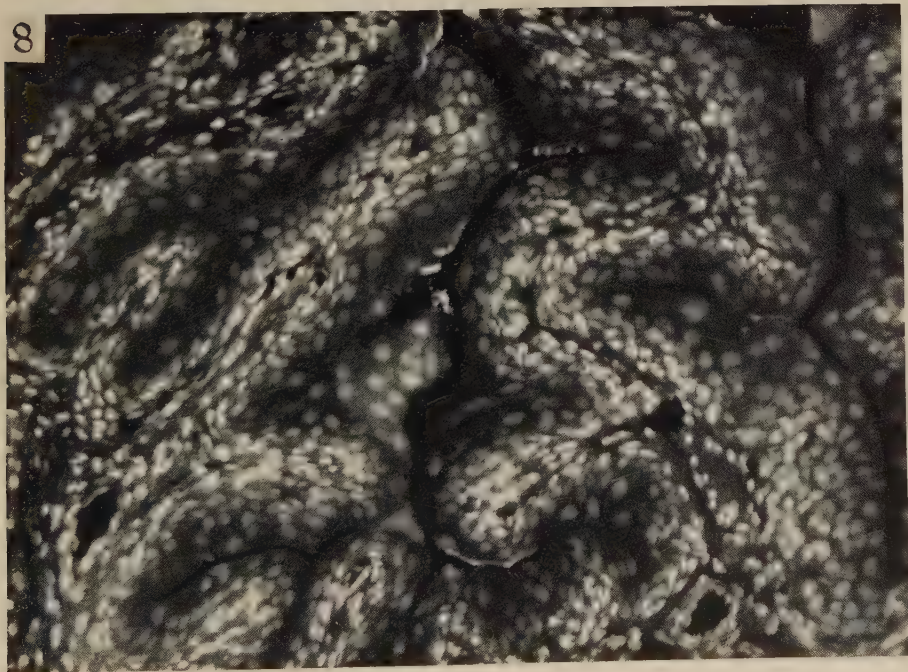
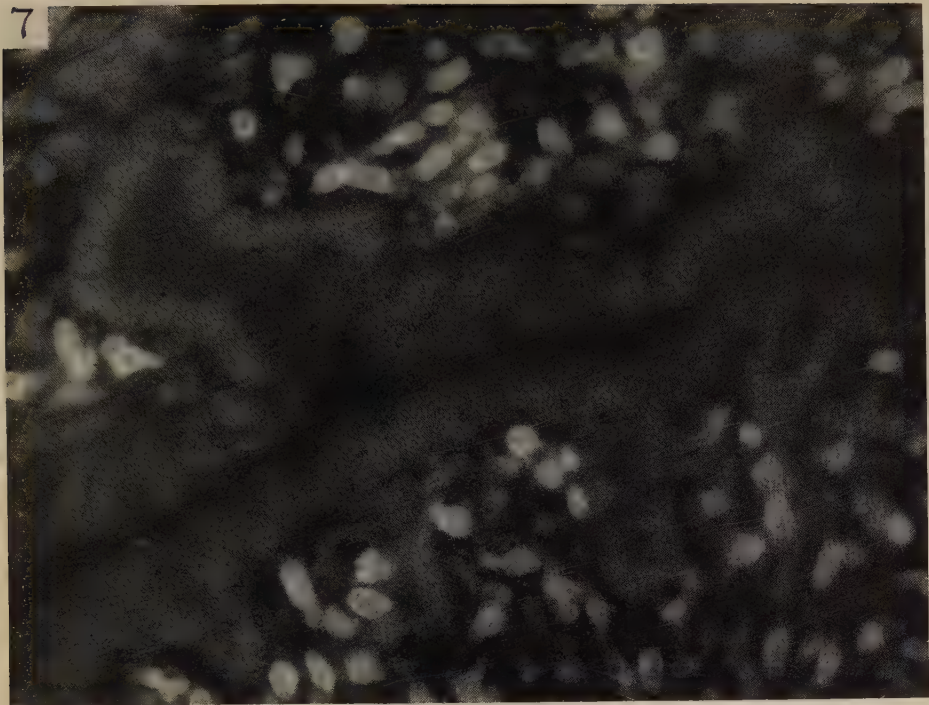
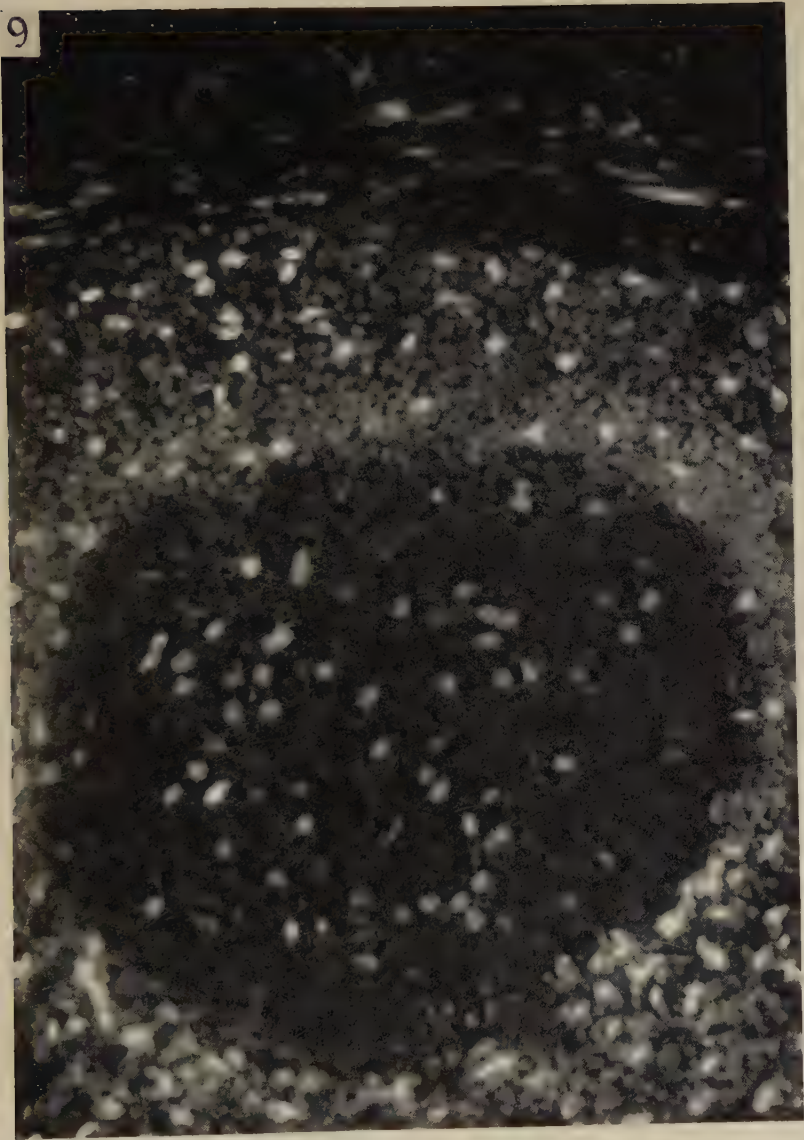


PLATE 4

EXPLANATION OF FIGURE

9 Lymph node, rabbit. Acriflavine HCl, 12.5 mg per kilogram, intravenous.  
Two hours after injection. Frozen-dried.  $\times 300$ .



## PLATE 5

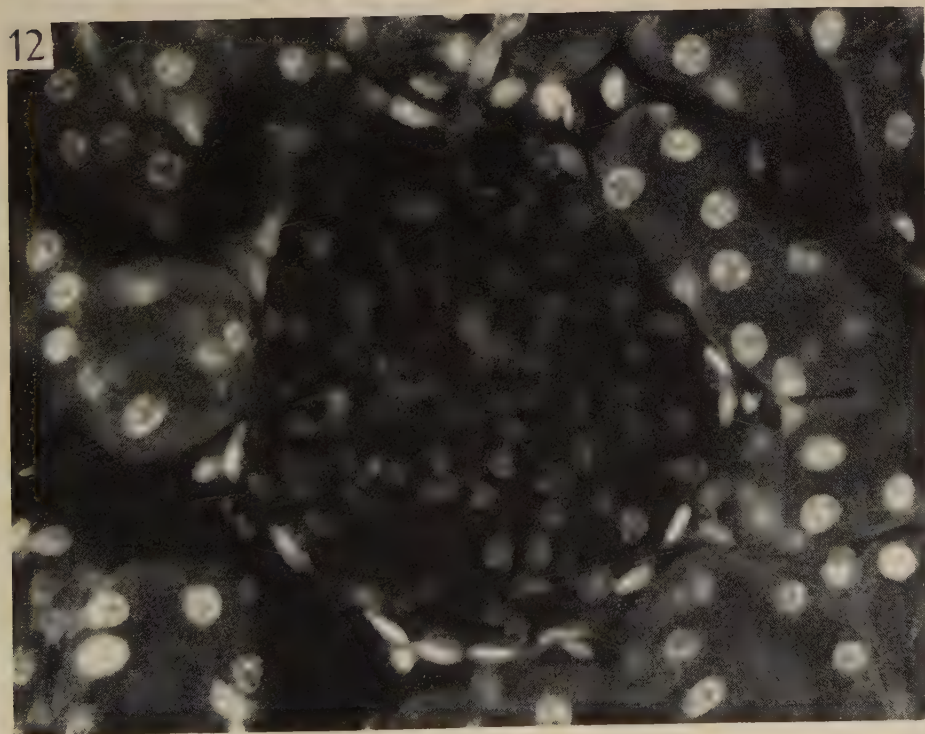
### EXPLANATION OF FIGURES

10 Tongue, rabbit. Acriflavine HCl, 12.5 mg per kilogram, intravenous. Two hours after injection. Note the bright fluorescence of mitoses in epithelium. Frozen-dried.  $\times 300$ .

11 Kidney, rat. Acriflavine HCl, 12.5 mg per kilogram, intravenous. Killed immediately after injection. At this time, the nuclei of the glomeruli stain brighter than those of the tubuli. Frozen-dried.  $\times 150$ .

12 Kidney, rabbit. Acriflavine HCl, 12.5 mg per kilogram, intravenous. Two hours after injection. The nuclei of the tubules stain brighter than the nuclei of the glomeruli at this interval. Frozen-dried.  $\times 600$ .





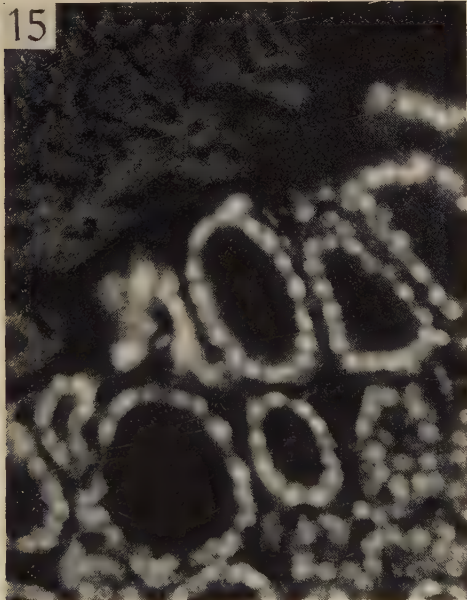
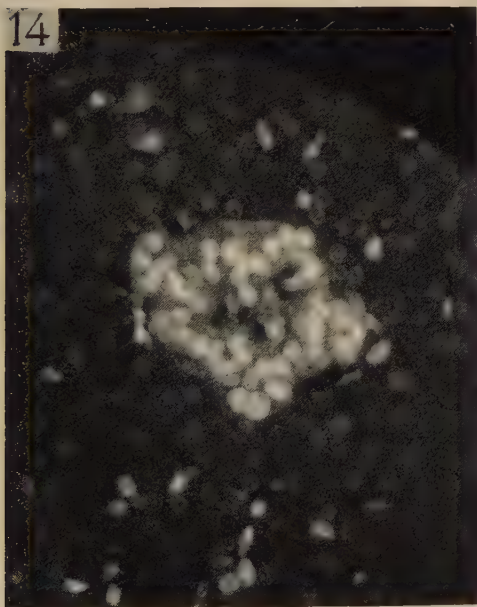
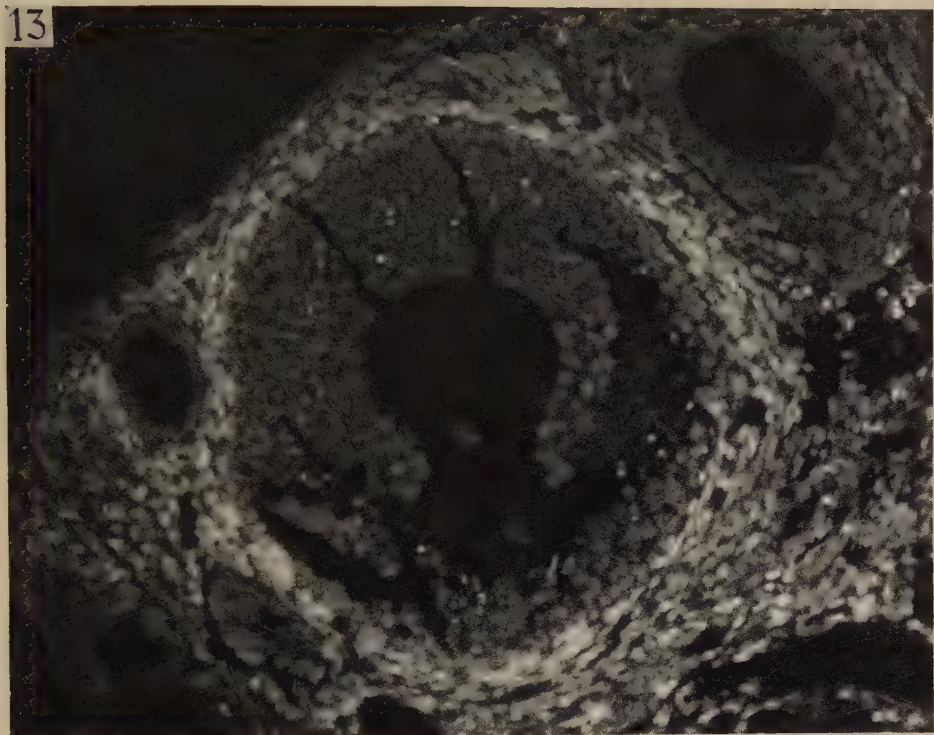
## PLATE 6

### EXPLANATION OF FIGURES

13 Ovary, mouse. Acriflavine HCl, 12.5 mg per kilogram, intravenous. One hour after injection. Frozen-dried.  $\times 260$ .

14 Pancreas, rabbit. Acriflavine HCl, 12.5 mg per kilogram, intravenous. Two hours after injection. Note bright fluorescence of islet nuclei. Frozen-dried.  $\times 300$ .

15 Thyroid and parathyroid, mouse. Acriflavine HCl, 12.5 mg per kilogram, intravenous. Two hours after injection. The nuclei of thyroid fluoresce with greater intensity than those of parathyroid. Frozen-dried.  $\times 300$ .







# SUBJECTION OF FETAL RATS TO SURGERY AND REPEATED SUBCUTANEOUS INJECTIONS: METHOD AND SURVIVAL <sup>1, 2</sup>

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TWO FIGURES

## INTRODUCTION

The observations to be reported grew out of experiments which were designed to determine whether the prenatal descent of the testis is governed in part by the action of androgen and whether the fetal testis is one source of this hormone of pregnancy. Using the rat, a species in which descent does not occur before birth, androgen was given by introducing it into the maternal circulation (Wells, '46c). The fetuses were usually smaller than the controls. Presumably the androgen had decreased the growth of the fetus by inhibiting the normal functioning of the placenta (Moore, '41a).

A second approach was the introduction of androgen into the amniotic sac (table 1, groups U and V). It is likely

<sup>1</sup> Aided by grants from: (1) the Committee on Human Reproduction, National Research Council, acting on behalf of the National Committee on Maternal Health, (2) the Ciba Pharmaceutical Products, Incorporated, (3) the Markle Foundation, (4) the American Philosophical Society and (5) the Medical Research Fund of the Graduate School.

<sup>2</sup> Author indebted: (1) to Drs. Ernst Oppenheimer, Fred Houghton and Cornelius H. Sullivan of the Ciba Pharmaceutical Products, Incorporated, for furnishing the testosterone propionate (Perandren) and the Trasentine hydrochloride, (2) to Dr. Dwight Ingle of the Upjohn Company for supplying the gonadotrophin (Gonadogen), (3) to Dr. John R. Mote of the Armour Research Laboratories for providing the adrenocorticotrophin, sample D 32, (4) to Mrs. Dorothy N. Highby for rendering expert technical assistance and (5) to Miss Virginia Moore for preparing the drawings.

that some of this hormone entered the fetal circulation via the extraembryonic vessels and fetal skin, yet it is not clear as to how much of it had been swallowed and then destroyed by the fetal liver (cf. the inactivation of androgen by the definitive liver, Biskind and Mark, '39; Biskind, '40; Danby, '40); in autopsying the fetuses of group V, droplets of oil were regularly found in the intestine and amniotic sac. Any hormone that reached the umbilical vein would have had to traverse either the hepatic sinusoids or the ductus venosus in order to enter the heart. (See ADDENDUM.)

The third approach was the transuterine injection of androgen under the skin of the fetus (group W). It soon became apparent that this is not a satisfactory method. By looking through the translucent uterus and fetal membranes, it was noted that much of the injected hormone promptly escaped into the amniotic sac as soon as the needle had been withdrawn.

Next it was decided to determine whether introduced gonadotrophin would cause descent of the testis. It was conceivable that it might do this by stimulating the testis to produce androgen in quantities sufficient to cause growth of the structures involved. The possibility of introducing the gonadotrophin into the maternal circulation was not given serious consideration because of the complication of the placental barrier. When gonadotrophin was injected into the amniotic sac, it did not necessarily kill the fetus (group X). How could we inject it under the fetal skin and then keep it from escaping into the amniotic cavity?

The first attempt led to rapid technical progress and, for this reason, some of the details should be of interest. One morning (May 21, 1944) a pregnant rat was etherized, and her peritoneal cavity was opened for the purpose of carrying out 4 steps of a new technique: (1) removing a fetus from the uterus in such manner that the placental circulation would not be interrupted, (2) injecting hormone under its skin, (3) sealing the site of puncture with thin celloidin and (4) returning the fetus and umbilical cord to the uterus.

All went well until the last step and it was a complete failure. The uterus had become greatly contracted. Attempts to stretch it with instruments resulted in tearing. As an improvisation, the uterine wound was sutured in two places in order to prevent an impending separation of the placenta. The fetus and its umbilical cord were placed in the peritoneal cavity of the mother. In a second fetus, the outcome was exactly the same. The maternal incisions were closed and the ether was discontinued. That evening the mother was given ether and her wound was opened. Both injected fetuses were alive and were given a second injection. Similarly, on the following day, morning and evening, these two fetuses were given 3rd and 4th injections. On the third day, at 5:00 P.M., 53 hours after the first injection, the mother was killed. Both fetuses were dead.

This degree of success pointed to the likelihood of attaining a second objective, that of subjecting fetuses to castration. Despite numerous failures, we eventually obtained two living fetuses which had been unilaterally castrated for a period of 48 hours (March 1, 1945).

The essentials of the technical procedure were abstracted (Wells, '46a) on the occasion of presenting at the Cleveland meeting of the American Association of Anatomists some observations on the secretion of urine by the fetal kidney (Wells, '46b). Meanwhile, we have added a series of short papers (Wells, '46c; Daly, Wells and Evans, '47; Hartmann and Wells, '48b), preliminary reports (Wells, '46d, '46e, '48) and abstracts (Wells, '47, '49; Hartmann and Wells, '48a; Fralick, '49; Sethre, '50).

The purpose of the present account is twofold: to introduce a companion paper (Fralick and Wells, '50) by bringing out the details of the principal elements of the technique and to record certain observations on survival. These details of the technique may be satisfactorily presented by concentrating upon the method of castrating fetuses, of implanting pellets of hormone and of making repeated injections

of hormones. As to the several variants of the basic technique, we shall deal with them in future papers.

With respect to the literature on methodology, we have recently found that fetuses had been subjected to surgery by Mayer ('15, '18) and Wolff ('19). Mayer ('15) ovariectomized an unborn puppy ("intrauterin kastrieren"). On the third day after the operation, the mother died of peritonitis. Later ('18), he made it clear that this had been a pregnancy of 6 weeks. After delivering this female fetus from the uterus, he had ovariectomized it and then placed it in the peritoneal cavity of the mother.

Wolff ('19) surgically produced some 47 secondary abdominal pregnancies in the rabbit, cat and rat by delivering the fetal membranes and the fetus which they enclosed. He did not remove these membranes. At least 10 of these rabbit fetuses lived, for periods of 1 to 7 days. Also, one of the cat fetuses lived until autopsy, 53 hours after the operation. In three of the rabbit fetuses, he had ligated either a hind leg or the neck after having withdrawn some of the amniotic fluid by means of needle and syringe. All survived (2 to 5 days). He decapitated one rabbit fetus *in utero*. At autopsy, 8½ hours later, this headless fetus was alive.

Before beginning our work we were familiar with certain contributions, notably those by Nicholas ('25a, '25b, '34), Bors ('25), Hooker and Nicholas ('30), Tobin ('39), Moore ('39, '41b, '41c, '43), Burns ('39a, '39b, '39c) and Moore and Bodian ('40).

Nicholas ('25b) used the fetal rat, opened the amniotic sac and amputated the forelimb, *in utero*. In 104 fetuses which were operated at 1 to 9 days before parturition 60 survived (58%). He reported that there was little danger of abortion except in the last two days before the close of gestation — "At this time an operation of any sort upon the mother is apt to bring about the premature birth of the litter." — p. 392. This is at variance with our observations, and doubtless is adequately explained by one of his sentences, p. 390, "The age of embryos, unfortunately, has



not been accurately timed." In 1934, he presented a second series of 104 fetuses in which the forelimb, or parts of it, had been amputated on the 14th to 20th day of pregnancy. The survival was about 70%.

Bors subjected fetal rabbits to surgery *in utero*. He opened the amniotic sac, ligated one leg and then amputated this extremity. When this operation was performed as early as the 19th day, he used a special apparatus to keep the fetus inside the uterus. Three fetuses in which the amputation was done on the 21st day lived until the time of autopsy, 6 days after the operation.

Hooker and Nicholas reported a series of 293 fetal rats in which the spinal cord had been sectioned *in utero*. At operation, the age ranged from 12 to 18 days "as determined either by inspection or by counting the number of days to parturition," (p. 419). The mortality was low, about 15%.

Tobin was able to destroy the adrenals of fetal rats *in utero*, on the 17th day, by using cautery. In 402 operated fetuses, the survival was about 40%. In three of those 160 which lived both adrenals had been completely destroyed (1.9%): This degree of success reflects the fact that the fetuses were quite young at the time of cauterization and that the adrenals must not have been clearly in view.

In such non-placental mammals as the opossum, the young in the pouch are not fetuses, yet their state of development is, in most respects, comparable to that of fetuses. They have been given inunctions and injections (Moore, '39, '41b; Burns, '39a, '39b, '39c, '45) and have been gonadectomized (Moore and Bodian, '40; Moore, '41c, '43).

Subsequent to the publication of our first reports (Wells, '46a, '46b), other workers have presented observations on fetal rabbits, mice and hamsters. Jost ('46a, '46b) used rabbits, and subjected fetuses to parabiosis, *in utero*. In the same species he gonadectomized 117 fetuses at the 19th to 24th day of life, gave certain individuals a pellet of androgen and terminated the experiments at the 26th to 28th day; 66 survived (56%); at the operation, only the caudal half

of a fetus was permitted to emerge from the uterus; after the gonadectomy had been completed, this exposed half of the fetus was returned to the uterus ('46c, '46d, '47a, '47b, '47c, '47d, '47f). He also decapitated fetuses ('47e).

Raynaud and Frilley destroyed either the gonads or the hypophysis of fetal mice, *in utero*, by irradiating locally a small area of the uterus (Raynaud and Frilley, '46, '47a, '47b; Raynaud, '49). This was successfully done as early as the 14th day of gestation, i.e., at 13 days and 9 hours.

Foote and Foote ('49) decapitated fetal hamsters and studied the effects of this hypophyseoprivia upon the thyroid. In a series of 141 fetuses in which the head was removed on the 12th day of pregnancy, 23 survived (16.3%).

#### FETUSES AND THE LENGTH OF GESTATION

Litters of fetal rats were usually obtained by mating albinos of the Sprague-Dawley strain, but were sometimes acquired by mating Sprague-Dawley males with piebald females. These females were from the colony of the Department of Biochemistry, St. Paul Campus. This colony had been developed from an initial cross between a wild Norway male and an albino female of the Jackson-Burr strain, a strain which was maintained for many years in the Department of Anatomy.

The beginning of pregnancy was dated from the exact time of witnessed coitus. Early in the work (1944), a female which had mated was not isolated from the males until the following morning. Since there were instances in which certain fetuses of a litter seemed to be considerably younger than the others (a point also noted by Nicholas, '42), the subsequent procedure was to segregate the female after a single ejaculation had occurred. While the number of fetuses per litter was somewhat smaller than before, the individuals of a litter were more uniform in size (age?); there were several cases of unilateral pregnancy. The matings were not scheduled at any special period of estrus — instead, any tested female which would accept the male was bred. Fe-

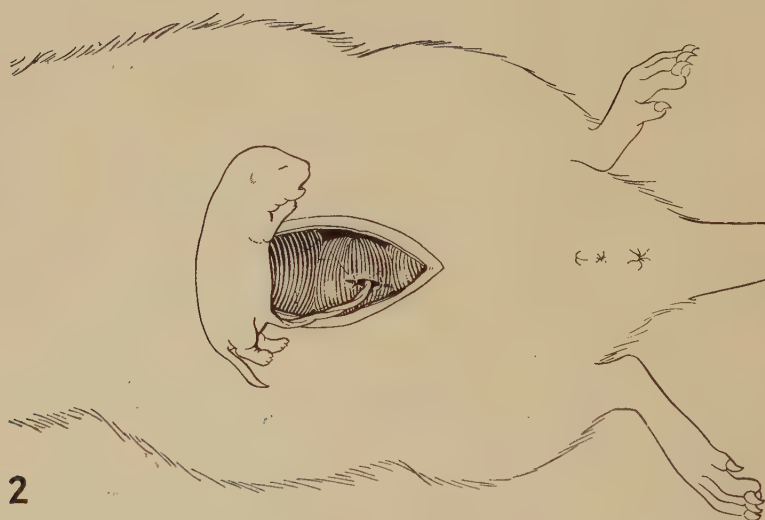
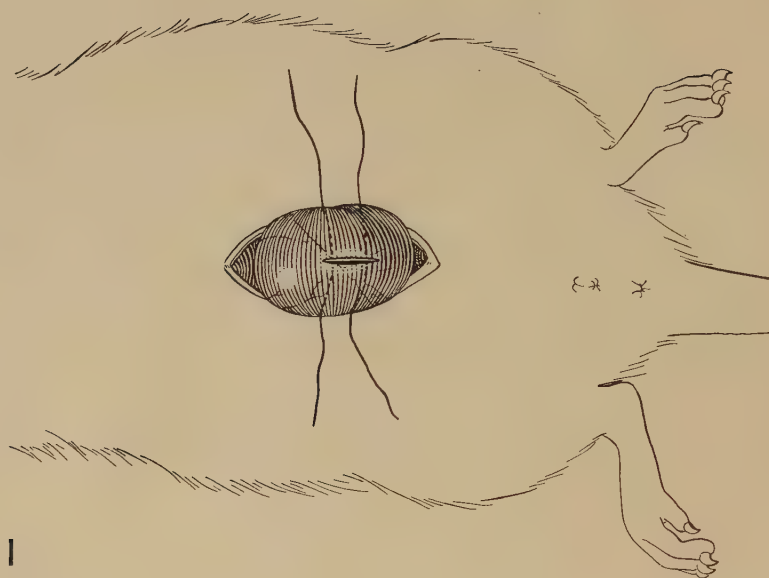
males were tested near midnight (between 9:45 P.M. and 2:00 A.M.) by placing individuals in a cage with several males.

The length of gestation was recorded in 23 cases of normal parturition (controls). These observations are being extended, and, in due time, will be published. For our present paper, the shorter observed periods are of greater interest than the longer ones, and, in 7 of the 23 cases, were approximately 21 days and 16 hours. Accordingly, those experimental fetuses which had been transferred to the peritoneal cavity of the mother and those which had been decapitated were usually taken by Caesarian section at 21 days and 15 hours after the coitus in order to prevent their death as a consequence of normal separation of the placenta.<sup>3</sup> Despite this precaution three fetuses were lost as a result of littering, one in group H and two in group K.

#### METHOD OF CASTRATION

The mother is etherized and prepared for midventral laparotomy by shaving the abdomen, starting cranially at the level of the xiphisternum and ending caudally at the last pair of nipples. Before planning the length of incisions, the over-all size of all derivatives of a fertilized ovum (fetus, membranes and placenta) is estimated by manual palpation. The skin is incised, beginning at the level of the next to last pair of nipples and extending upward about 30 mm. The peritoneal cavity is opened at the linea alba. The latter incision should be ample for delivering that portion of a uterine horn which contains a single fetus, but should not be excessively long (usually about 25 mm). The next to last fetus in one horn (second fetus above the cervix) is located by inspection. The portion of uterus containing this fetus is delivered through the wound with the aid of blunt forceps

<sup>3</sup> The reckoning of a particular day of pregnancy should not lead to confusion. In group A, for example, treatment was usually begun on the 20th day; the median of the group (20th day) was about 19 days and 9 hours after the coitus, while the earliest of the range (19th day) was about 18 days and 10 hours after it.



Figures 1 and 2



or of slight manual pressure upon the abdominal wall or of both. If the wound is optimal with respect to length, it serves the useful purpose of inhibiting the spontaneous return of this exposed uterine segment.

The procedure for delivering the fetus without causing any separation of the placenta is as follows. The translucent, antimesometrial aspect of the exposed uterine segment is explored for the purpose of choosing the best site for an incision, namely the site which lacks large uterine vessels and subjacent extraembryonic vessels. In advance of cutting, the uterine wall is underlaid with two threads which are placed transverse to the long axis of the uterus and about 6 to 10 mm apart (fig. 1). These threads may be either buttonhole silk or no. 100 mercerized cotton. Using iridectomy scissors, the uterine wall is longitudinally incised. Cutting is begun about 3 mm below the lower thread and ended about 3 mm above the upper one. The skin of the fetus is exposed by tearing the fetal membranes with jeweler's forceps. In doing this, all but the smallest extraembryonic vessels should be spared. After the emergence of the amniotic fluid, the uterus exhibits spontaneous but slow contraction. Either the cranial or caudal end of the fetus is introduced into the uterine wound, between the threads, by applying slight manual pressure upon one end of the exposed uterine segment and by manipulating the threads with forceps. The gradual contraction of the uterus tends to accelerate the delivery of the fetus. As a precaution against speedy emergence, both ends of one thread are grasped, and a large loop for a first knot is prepared in advance. Upward tension on this loop slows down the emergence of the fetus. The fetus is delivered by momentarily relaxing this tension (left hand) and by applying slight digital pressure (right hand). A double knot is quickly tied, and both ends of the second thread are promptly grasped in order to prevent the emergence of any portion of the placenta. This thread is also securely tied after making sure that neither the umbilical cord nor any extraembryonic vessel is likely to be caught

in the knot. The uterine segment and contents (placenta and extraembryonic membranes) are returned to the peritoneal cavity of the mother. At the conclusion of the foregoing steps the placental circulation is intact, the umbilical cord traverses an ample uterine window and the fetus lies on the shaved skin of its mother (fig. 2).

We should digress to explain that the drawings were obtained from a near-term pregnancy and that the operation is usually performed on the 20th day (at about 19 days and 9 hours after the coitus). Accordingly, the fetus would be much more immature than the one illustrated.

If the exposed fetus turns out to be a female, its umbilical cord is ligated and severed, and it is removed. Search for a male is continued, by delivering, as before, one of the two adjacent individuals, usually the one which is farthest from the maternal cervix uteri. If this fetus is a female, the search is extended to the opposite uterine horn.

In preparing a male for castration, it is placed lengthwise in the abdominal wound of the mother (not illustrated). It lies on its back and its head is at the cranial end of the wound. Since etherization of the mother to a safe stage of surgical anesthesia does not satisfactorily inactivate the fetus, undesirable fetal movements may be overcome by giving the fetus an injection of nembutal or, preferably (to save time), by restraining it with two blankets of saline-moistened cotton. The largest blanket of the two is designed to cover the umbilical cord, most of the fetus (including the snout) and large areas of the shaved maternal skin. It is first brought into contact with the fetal skin, and used as a means of rotating the fetus into the exact position desired, viz. with the left flank uppermost. Then, while this blanket is still dripping wet, its two ends are plastered to the skin of the mother. The hind legs and tail of the fetus are similarly restrained by applying the smaller blanket. When this process of draping has been completed, only the urogenital papilla and the left flank are exposed. This

blanketing serves the twofold purpose of restraining the fetus and of moistening it.

After placing the mother in the position illustrated, the bare skin of the left flank of the fetus is viewed through the wide field of a Bausch and Lomb binocular dissecting microscope. The microscope is equipped with  $\times 0.7$  objectives, with  $\times 15$  oculars, with a Nicholas illuminator and with a Huber base.

Next the peritoneal cavity of the fetus is opened by making high, oblique, inguinal incisions. The skin is incised with iridectomy scissors, beginning below at a point which is outside the inferior epigastric vessels and extending obliquely upward and outward as far as the region of the spleen. Using jeweler's forceps, the external oblique layer is exposed by reflecting the overlying tela subcutanea. With forceps in the left hand and scissors in the right, the external oblique layer is grasped and the several uncut layers of the abdominal wall, muscular and peritoneal, are simultaneously incised. This second incision need not necessarily be as long as the first.

The left testis is exposed by reflecting such viscera as the pancreas, spleen, small intestine and bladder. This is done with the aid of two tiny cotton spindles which are prepared by cutting into thirds a no. 3 Richmond dental pellet, by moistening the pieces in saline and by rolling them individually between forefinger and thumb. With forceps in both hands, these two spindles are individually grasped and then used as retractors. After the testis and epididymis are brought into view, the retracted viscera are prevented from returning to their original position by tucking both cotton spindles underneath the medial flap of the deep incision and by leaving these spindles in place, temporarily, in the opened peritoneal cavity.

In removing the left testis, two forceps are the only instruments used, and neither the testis nor the epididymis is grasped. Instead the testis is shelled out of the epididymis by tearing the intervening "mesentery" and contents. Bleed-

ing from such vessels as the internal spermatic is only momentary. The vessels are not ligated.

After removing the cotton spindles, the two incisions are individually closed by suturing. Two uninterrupted sutures per incision are usually adequate. In suturing, the left hand uses forceps and the right hand uses a straight needle which is threaded with an unravelled strand of buttonhole silk. This strand must be repeatedly moistened in order to prevent it from tearing (sawing) the flaps of the incision.<sup>4</sup>

To remove the right testis, the fetus is rotated into that position which brings the right flank uppermost, and is re-draped with new blankets of moistened cotton. It is more difficult to remove the right testis than the left. The main complication is that the lower part of the right lateral lobe of the liver must be retracted upward. The liver is fragile!

At the conclusion of the operation, the fetus is unblanketed and introduced into the maternal peritoneal cavity. It is placed on its back in that lateral "gutter" which is between the uterine horn and the abdominal wall. Its belly should face the surgically produced uterine window in order to avoid any unnecessary stretching and kinking of the umbilical cord. A second fetus of the litter may be similarly delivered and castrated provided the sex ratio does not militate against this.

The mother's inner abdominal incision is closed with continuous sutures, while her skin incision is clamped with two or three no. 11 Michel wound clips. The ether is discontinued.

During the foregoing operation, it is next to impossible to maintain a bacteriologically sterile technique since it is not practical to wear rubber gloves and to drape the maternal wound with sterile towels. As precautions against sepsis, however, the instruments are sterilized in steam, for 15 minutes, and the shaved skin of the mother and the hands of the operator are washed with soap and water. Even if

<sup>4</sup>In two operated fetuses, incidentally, the inguinal wound was inadequately sutured; both lived until autopsy, 61 hours after the castration, but had intestinal loops which had herniated and which showed distention and inflammation (?).



a strictly sterile technique were attempted, it is likely that it could not be maintained throughout the course of a series of injections, as, for example, in groups Q and S.

#### METHOD OF IMPLANTING A PELLET OF HORMONE

A delivered fetus is placed lengthwise in the abdominal wound of the mother, with its back uppermost (not illustrated). This keeps the umbilical cord from drying. The fetus need not be blanketed with moist cotton, but its skin should be repeatedly moistened with saline. Its skin is so thin that it need not be incised for the purpose of implanting a pellet, but instead may be punctured with closed jeweler's forceps.

The skin is punctured in the mid-dorsum of the lumbar region, and the sharp tip of the closed forceps is extended upward, underneath the skin, as far as the vertebral angle of the scapulae. The subcutaneous tunnel thus produced is widened by allowing the jaws of the forceps to open a bit. After withdrawing the closed tips of this instrument, a weighed pellet is introduced into the tunnel. The puncture wound is closed with a single suture, and the fetus is placed in the peritoneal cavity of the mother.<sup>5</sup>

#### METHOD OF MAKING REPEATED SUBCUTANEOUS INJECTIONS

Those hormones which do not freely cross the placental barrier may be introduced into the fetal circulation by injecting them under the fetal skin. Since it is likely that they are metabolized in part and excreted in part, the maintenance of a continuous supply requires repeated injections.

A delivered fetus is placed lengthwise in the abdominal wound of the mother, with its back or one side uppermost (not illustrated). This prevents drying of the umbilical cord.

<sup>5</sup> Dr. Ralph L. Kitchell, who is studying the effects of introduced hormones upon certain fetal glands including the adrenal and also the effects of surgical adenoprivia, has implanted pellets of hormone under the fetal skin *in utero* (unpublished).

The fetus need not be immobilized by blanketing, but should be kept moist until the time of inserting the needle.

The hormone to be injected is drawn up into a 0.25 cm<sup>3</sup> syringe and its attached one-half inch needle, no. 27. While the quantity to be given at a single injection is usually 0.05 cm<sup>3</sup>, twice this amount may be injected. The latter amount, however, makes a very large blister.

The skin of the neck is punctured, and the full length of the needle is buried by directing its point caudally. The point usually reaches the lumbar region. The plunger is pushed and the needle is allowed to remain temporarily in place. The site of puncture is to be sealed with celloidin, and, in doing this, the surrounding skin must not be wet. Accordingly, that long cutaneous ridge which is made by the buried needle is lightly clamped with blunted jeweler's forceps during the process of withdrawing the needle. The points of the forceps are placed astride both ridge and needle, in advance, and are promptly closed after the point of the needle has passed them. A small droplet of thin celloidin is applied, and is permitted to spread and dry. Then the clamping is discontinued.

The injected fetus is placed in the peritoneal cavity of the mother, and a second fetus is injected. After the abdominal wound of the mother has been sutured and clamped, the etherization is stopped.

In making the succeeding injections of a series, the mother is etherized and her peritoneal cavity is reopened. Any adhesions which bind the two injected fetuses to the maternal viscera or which are tending to strangulate the two umbilical cords, are broken with blunt forceps. These fetuses are successively brought into the maternal wound and injected, as before. If one fetus is dead, it is removed by severing its umbilical cord. The individual injections of a series are usually made about 12 hours apart.

#### SURVIVAL AND MORTALITY

Table 1 shows that 853 listed fetuses lived until autopsy (59%) and that 582 did not (41%). The question arises

| GROUPS                                                                                                     | LITTERS | FETUSES | TREATMENTS                                              |                        | INJECTIONS <sup>1</sup> | FETUSES ALIVE AT END OF EXPERIMENTS |          |                                          |
|------------------------------------------------------------------------------------------------------------|---------|---------|---------------------------------------------------------|------------------------|-------------------------|-------------------------------------|----------|------------------------------------------|
|                                                                                                            |         |         | Type                                                    | Day begun <sup>1</sup> |                         | Number                              | Per cent | Hours after first treatment <sup>1</sup> |
| Series I. Fetuses removed from uterus and given subcutaneous injections                                    |         |         |                                                         |                        |                         |                                     |          |                                          |
| A                                                                                                          | 42      | 80      | Gonadotrophin                                           | 20 (19-20)             | 4 (3-6)                 | 29                                  | 31       | 53 (38-72)                               |
| Ac                                                                                                         | 7       | 7       | Uninjected littermates (controls)                       | 20                     |                         | 2                                   | 29       | 43 (33-51)                               |
| B                                                                                                          | 27      | 53      | Adrenocorticotrophin                                    | 20                     | 4 (1-5)                 | 16                                  | 30       | 48 (14-54)                               |
| C                                                                                                          | 10      | 21      | Androgen in oil                                         | 20 (19-20)             | 3 (3-4)                 | 4                                   | 19       | 42 (42-46)                               |
| Series II. Fetuses removed from uterus and subjected to surgery                                            |         |         |                                                         |                        |                         |                                     |          |                                          |
| D                                                                                                          | 17      | 18      | Laparotomy ("sham" operation)                           | 20                     |                         | 6                                   | 33       | 52½ (51-53)                              |
| E                                                                                                          | 17      | 19      | Unilateral castration                                   | 20                     |                         | 11                                  | 58       | 51 (47-56)                               |
| F                                                                                                          | 40      | 56      | Castration                                              | 20 (19-20)             |                         | 21                                  | 39       | 52 (20-61)                               |
| Fc                                                                                                         | 40      | 19      | Unoperated littermates (controls)                       | 20                     |                         | 7                                   | 37       | 53 (29-61)                               |
| G                                                                                                          | 5       | 7       | Adrenalectomy                                           | 20                     |                         | 3                                   | 43       | 51 (51-56)                               |
| Gc                                                                                                         | 6       | 6       | Unoperated littermates (controls)                       | 20                     |                         | 2                                   | 33       | 50 (49-51)                               |
| H                                                                                                          | 16      | 19      | Castration and adrenalectomy                            | 20 (19-20)             |                         | 6                                   | 32       | 45 (36-51)                               |
| Hc                                                                                                         |         | 12      | Unoperated littermates (controls)                       | 20                     |                         | 10                                  | 83       | 50 (36-52)                               |
| I                                                                                                          | 3       | 6       | Cautery of left ureter                                  | 20                     |                         | 3                                   | 50       | 43 (43-54)                               |
| I                                                                                                          | 31      | 62      | Ligation of left ureter                                 | 20 (18-21)             |                         | 42                                  | 68       | 50 (21-59)                               |
| J                                                                                                          | 9       | 17      | Cautery of urogenital papilla                           | 20 (20-21)             |                         | 10                                  | 59       | 26 (24-33)                               |
| K                                                                                                          | 9       | 17      | Ligation of ug. pap. (2 + fetuses/litter) <sup>2</sup>  | 21 (17-22)             |                         | 205                                 | 95       | 2 (1-52)                                 |
| L                                                                                                          | 57      | 216     | Ligation of ug. pap. (many fetuses/litter)              | 21 (20-22)             |                         | 332                                 | 76       | 21 (5-27)                                |
| M                                                                                                          | 49      | 437     |                                                         |                        |                         |                                     |          |                                          |
| Series III. Fetuses subjected to surgery <i>in utero</i>                                                   |         |         |                                                         |                        |                         |                                     |          |                                          |
| N                                                                                                          | 25      | 62      | Decapitation                                            | 19 (17-21)             |                         | 21                                  | 34       | 71 (22-102)                              |
| Series IV. Fetuses removed from uterus, subjected to surgery and given subcutaneous injections or implants |         |         |                                                         |                        |                         |                                     |          |                                          |
| O                                                                                                          |         | 13      | Castration and androgen (pellets)                       | 20                     | 1                       | 7                                   | 54       | 51 (51-52)                               |
| P.                                                                                                         | 13      | 12      | Castrated littermates (controls)                        | 20                     |                         | 11                                  | 92       | 51 (50-53)                               |
| Q                                                                                                          | 24      | 34      | Decapitation and gonadotrophin (injections)             | 20 (19-20)             | 5 (4-5)                 | 6                                   | 18       | 50 (47-53)                               |
| R                                                                                                          | 24      | 23      | Decapitated littermates (controls)                      | 20                     |                         | 9                                   | 39       | 50 (24-53)                               |
| S                                                                                                          | 29      | 42      | Decapitation and adrenocorticotrophin (inj.)            | 20                     | 4 (4-5)                 | 9                                   | 21       | 51 (42-54)                               |
| T                                                                                                          |         | 33      | Decapitated littermates (controls)                      | 20                     |                         | 12                                  | 36       | 51 (42-54)                               |
| Series V. Injections made without removing fetuses from uterus                                             |         |         |                                                         |                        |                         |                                     |          |                                          |
| U                                                                                                          | 11      | 83      | Androgen in methocel introduced into the amniotic sac   | 20 (16-21)             | 2 (1-3)                 | 35                                  | 42       | 48 (21-74)                               |
| V                                                                                                          | 9       | 40      | Androgen in oil introduced into the amniotic sac        | 19 (17-20)             | 1½ (1-2)                | 16                                  | 40       | 85 (55-96)                               |
| W                                                                                                          | 7       | 26      | Androgen in oil introduced under the skin of the fetus  | 20 (19-20)             | 2 (2-3)                 | 10                                  | 38       | 55 (55-79)                               |
| X                                                                                                          | 2       | 12      | Gonadotrophin solution introduced into the amniotic sac | 19½ (19-20)            | 5 (4-6)                 | 8                                   | 67       | 54 (36-71)                               |
|                                                                                                            | 443     | 1435    | Totals                                                  |                        |                         | 853                                 | 59       |                                          |

<sup>1</sup> Median or theoretical median of a group. Numbers in parentheses indicate the range.<sup>2</sup> Ug. pap., urogenital papilla.

as to whether the adrenocorticotrophin and gonadotrophin caused the death of certain fetuses by acting as toxic substances. It is impossible to arrive at a satisfactory answer without the benefit of data on the effects of a series of control injections. In fact, in considering those factors which contributed to our over-all mortality of 41%, the evidence similarly is largely circumstantial.

The death of 143 of the 582 fetuses (25%) was probably due in part to the fact that the treatment was begun prior to the 20th day. Twelve of these were members of group A, 5 of group C, 2 of F, 2 of H, 2 of J, 5 of L, 39 of N, 47 of U, 23 of V, 4 of W and 2 of X. On the other hand, the commencement of treatment prior to the 20th day did not always lead to the death of the fetus. In 56 of those fetuses which lived, in fact, the treatment was begun on the 17th to 19th day (2 in group A, 4 in L, 16 in N, 16 in U, 12 in V, 2 in W and 4 in X). In groups N, U, V, W and X, of course, the treated fetuses remained in the uterus.

The death of 59 of the 582 fetuses (10%) was associated with the death of 13 of the 443 mothers (2.9%). These losses were as follows: group A, 1 mother and 2 fetuses; groups D and F, 1 mother and 2 fetuses; group H, 3 mothers and 5 fetuses; group M, 4 mothers and 36 fetuses; group N, 1 mother and 4 fetuses; groups Q and R, 1 mother, 1 injected fetus and 1 uninjected fetus; groups S and T, 2 mothers, 2 injected fetuses and 6 uninjected fetuses. At autopsy, one of these 13 mothers was found to have died of peritonitis (groups S and T). Presumably peritonitis had also killed the 12 other mothers but it was not possible to determine this point because a number of hours elapsed between these particular deaths and autopsies.

Six of the 582 fetuses (0.01%) were lost by 4 cases of littering: group F, 1 fetus; group H, 1 fetus; group K, 2 fetuses; group N, 2 fetuses. In these 4 cases of littering, the approximate time after the coitus was, respectively, 21 days and 19½ hours, 21 days and 14½ hours, 21 days and



12½ hours and 21 days and 17 hours. This leaves 374 of those 582 fetuses which died (64%).

In series III the high mortality was almost exclusively due to the decapitation, while in series V the deaths are only of passing interest. In series I, II, and IV, it would seem that the mortality in the several groups was more or less proportional to the number, frequency, severity and duration of those special hazards of life which had been experimentally introduced.

Thus, in concentrating upon series I, II and IV, note that the highest survival in an individual group was 95% (group L). This no doubt reflects the fact that in group L only a few fetuses per litter were used, that they were subjected to a single treatment (not repeated), that this treatment was relatively mild and that the period of treatment was comparatively short.

It is clear that the use of many fetuses per litter increased the hazards. In group M, for example, a group in which the duration of the experimental period was about one day, we used 8.7 fetuses per litter (average). The survival was 76%. At autopsy it was sometimes observed that the umbilical cords of two fetuses were entwined and were kinking each other. If two or three treated fetuses had died, it was rare to find the others alive. This suggests that toxins from necrotic fetuses contributed to the death of treated littermates in group M and in other groups as well. That such toxins in the peritoneal cavity of the mother may have been swallowed by living fetuses, is suggested by the following observation: in autopsying those treated fetuses which had lived, it was regularly noted that the rectal meconium was bloody. Despite the hazards of using many fetuses per litter, however, group M includes 9 litters in which all the treated fetuses lived.

When sex and littermate controls were not limiting factors in an experiment, it was possible to use the first two fetuses which were taken out of the uterus, one from each uterine horn. This was usually done in group J and the survival

was 68%. If two fetuses of a litter of the 18th day be excluded from group J, there were in this group only 4 cases of loss of both fetuses. There were 13 cases in which two of two survived and one case in which three of three lived.

In most groups the search for males and the use of two or more males per litter required the surgical delivery of several fetuses per litter, including females. This probably contributed to the mortality. In each of 14 groups, the average number of delivered fetuses per litter was as follows: A, 5.5; A<sub>c</sub>, 5; D, 3; E, 3; F, 4; F<sub>c</sub>, 5; G, 6; G<sub>c</sub>, 6; H, 3; H<sub>c</sub>, 3; O, 4.3; P, 4.3; Q, 4; R, 4.

In group B, however, all delivered fetuses, regardless of sex, were injected. The average number per litter was two. Similarly, in groups S and T, both males and females were used, but in an individual litter the injected fetus and its decapitated control were of the same sex. To obtain them from the uterus it was sometimes necessary to deliver three fetuses, the average number per litter being 2.6.

It is likely that one of the causes of death was strangulation of the umbilical cord. In those fetuses which were given repeated injections, for instance, an impending strangulation was sometimes noted. It was being caused either by the peristalsis of a loop of maternal intestine or by annular adhesions.

The survival was relatively low in all groups in which repeated injections were given. This reflects the fact that the introduced hazards were indeed numerous and great. That momentary drying of the fetal skin which was a prerequisite for sealing the site of puncture with celloidin must have contributed to the formation of adhesions (strangulation of the cord). The poor survival in group C (19%) was doubtless due in part to the fact that these injections were made at a time when the technique was being developed ('44).

#### DISCUSSION

The basic technique permits a direct experimental approach to a number of problems in that fascinating field

of investigation, the physiology of the fetus. There are certain distinct advantages in using the rat. An abundance of living material may be readily obtained at not too great an expense. Also, as is commonly known, the rat has an unusually high resistance to infection. Furthermore, the fetus is relatively immature at term. In fact, the embryonic primordia of such organs as the prostate and seminal vesicles appear only a few days before birth. In series I, II and IV, 25 fetuses tolerated various experimental treatments for 54 hours or longer. A period of 54 hours is about 10% of the total period of pregnancy.

It may be possible to develop modifications which would allow us to return the operated or injected fetus to the uterus.<sup>6</sup> This should increase the survival and make it worthwhile to begin the experiments at still earlier stages of pregnancy. In this connection, note that fetuses were decapitated *in utero* and that the longest period of survival was 102 hours (group N). This is about one-fifth of the total period of gestation.

Even if it is possible to improve the basic technique, however, there are certain factors which will undoubtedly militate against extending the direct experimental approach backward as far as the 17th day. These younger fetuses have such immature tissues that they should not be expected to withstand handling, surgery and subcutaneous injections. The uterine wall is exceedingly thin at the 17th day. The physiology of the gravid uterus and of the placenta, at this time, may also be a limiting factor. In fact, it would seem that this is a critical period in the survival of normal fetuses.

Reynolds ('46) studied the growth of the gravid uterus, especially in the rabbit, and correlated the manner of growth with the growth and welfare of the fetus. He noted that the curve of uterine growth is sigmoid. During the period

<sup>6</sup> Working with Mr. Arthur E. Sethre, our attempts have largely involved the use of such antispasmodic drugs as Ciba's Trasentine hydrochloride for the purpose of reducing that slow contraction of the uterus which works against a successful return — Jost ('47d) used Spasmalgine.

of most rapid uterine growth, the uterus at the site of individual conceptions is spheroidal. Later the rate of growth declines and this spheroid is converted into a cylinder. He advanced the working hypothesis that tension on the uterine wall attains a maximum value at the time when the spheroid is maximal, that this tension causes locally a transient physiological ischemia in the uterine vessels, and that the ischemia is relieved as soon as the conversion from spheroid into cylinder takes place. He noted that fetal death, as evidenced by resorption, most commonly begins to be encountered near the end of the period of the spheroid and that the conversion into a cylinder occurs at about the 20th day. After conversion has occurred, the viability of the fetus is relatively assured. Later, Reynolds presented additional observations which support his several working hypotheses ('49a, '49b).

In the rat this conversion occurs on the 18th day of pregnancy (Bradin, '48). Thereafter, the fetus spends about 18% of the total period of intrauterine life in an elongating uterus in which the conditions for fetal growth are optimal (Reynolds, '49b). Hamster fetuses spend about 10% of the total period, rabbit fetuses about 28%, monkey fetuses about 39% and guinea pig fetuses about 55%.

It is likely that these conditions for optimal fetal growth include an increased permeability of the placenta. Flexner and Pohl ('41) studied the permeability of the placenta of the rat by measuring the rate of transfer of injected radioactive sodium. Their figure 5 shows that there is a rapid increase in permeability at about the 18th day.

In summary, we may be able to make important improvements in our technique, but should not expect any large measure of success in subjecting fetal rats to laparotomy and subcutaneous injections as early as the 17th day of prenatal life. Prior to the 18th day, the limiting factors include immaturity of the fetal tissues, thinness of the uterine wall and lack of those special uterine and placental conditions which favor optimal growth and survival of normal fetuses.



## ADDENDUM

With respect to the suggestion that swallowed androgen might have been inactivated by the liver (p. 310), we now have evidence that "The liver does not inactivate estrogens in the monkey (*Macaca mulatta*)" and that "In this particular, the primate is unlike the rodent whose physiological responses form the basis for so much of the present thinking" (Van Wagenen and Gardner, '50, p. 272).

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## BOOK REVIEWS

GENERAL CYTOLOGY. By E. D. P. DE ROBERTIS, W. W. NOWINSKI, AND FRANCISCO A. SAEZ, translated into English by Warren Andrew. 1948. xi + 345 pages, 143 figures. \$5.50. W. B. Saunders Company, Philadelphia. (Spanish edition, Argentina, 1946.)

The authors are active investigators in the field of experimental cytology. De Robertis is perhaps the most omnivorous of the three in his interest in the activities of the cell and has published contributions especially in cytochemistry. Nowinski is particularly interested in the living cell in its physicochemical aspects and its metabolic activity. Saez has specialized in the nucleus, chromosomes and cytogenetics.

The book arose originally from a desire to provide a synthesis of modern cytology in the Spanish language. The success of the Spanish edition has prompted the undertaking of this English edition which is much more than a mere translation. The scope and plan were not altered but much new material and thirty-four illustrations were added.

Chapter I presents an introduction to the problem of the organization of living matter and a short historical summary leading to the cell theory and the Protoplasm Theory. Chapter II gives the chemical and physico-chemical foundations of the structure and function of the cell. Chapter III describes the general microscopic organization of the cell. Chapter IV considers the submicroscopic organization or ultrastructure of protoplasm. Chapter V deals with the structure, composition and functional significance of the cytoplasmic organoids. In Chapter VI the molecular structure of the plasma membrane and the phenomena of permeability are considered. Chapter VII describes the structure and chemical composition of the nucleus. Chapter VIII discusses the morphology and internal organization of the chromosomes and their behavior in the process of cell division; and Chapter IX correlates the chromosomes and genetic phenomena (cytogenetics). In Chapter X the enzyme systems which take part in metabolism and respiration in the cell are described.

In Chapter XI the visible manifestations of cellular activity, and in Chapter XII the phenomena of differentiation, senescence and death of the cell, are considered.

It is not possible to review the material included in this book because it is already very meaty and condensed. In each chapter the authors adhere closely to their subject. The information is carefully presented, clearly stated and given adequate authenticity by quotations and citations from published work. Each chapter is followed by a list of approximately 40 references.

It is more interesting and pertinent to point out the acknowledged purposes and philosophy of the authors and the implications provided by their treatment. In the preface there is the statement, "In the present book an attempt has been made to stress the morphological, physiological and genetic aspects of modern cytology." This is a bold pronouncement of wishful thinking on the part of incurable experimentalists. They do start out with a sound coverage of classical cytological tenets but seem to be restless to move on and soon find themselves hopelessly involved in physics, cytochemistry or new and quantitative techniques. Again they pledge allegiance to the morphological and functional aspects of cytology as well as the dynamic, but it is quite clear to the reader that dynamic phases are of paramount interest in almost every instance.

It is gratifying to see that cytology includes more than the study of the nucleus and the chromosomes. Three of the 12 chapters deal with the nucleus, however, and the longest chapter in the book, 50 pages, gives a good summary of cytogenetics. The authors extend cytology beyond classical lines in chapters devoted to such fundamental but commonly slighted subjects as the plasma membrane and cell permeability, enzymes and cell respiration, visible manifestations of cellular activity, and differentiation, senescence and death of the cell.

The authors recognize the association between technical advancement and theoretical progress by inserting descriptions of appropriate new techniques and apparatus. Cytochemistry is not given a separate chapter but is mentioned frequently throughout the book.

The book is effectively but not profusely illustrated, the printing and binding are good and there is a careful and reasonably complete index. The translator should shine by something more than reflected light for his clear and readable style. Every student of histology or cytology should find this book a valuable tool as well as a stimulating source of information.

C. M. G.

RADIOGRAPHIC ATLAS OF SKELETAL DEVELOPMENT OF THE HAND AND WRIST. By WILLIAM WALTER GREULICH and S. IDELL PYLE. 1950. xiii + 190 pages, illustrated. \$10.00. Stanford University Press, Stanford, California.

Doctor Greulich is Professor of Anatomy at Stanford University, a position which he took following a period as Professor of Anthropology and Anatomy and Director of the Brush Foundation at Western Reserve University. He has contributed many publications in the fields of skeletal development and growth toward maturity. Miss Pyle is a Research Associate at Western Reserve University and has had practical experience with growth problems at the Spies Nutrition Clinic of Hillman Hospital, Birmingham, Alabama.

The purpose of this Atlas is to provide a series of x-rays of the hand and wrist as standards of skeletal development which may be used to determine the developmental status of a child. It was written in response to the need, emphasized by the demands of well-baby clinics and school health programs, for more precise and less variable measurements than height, weight and age.

The material on which the Atlas is based is a Research Series begun under the leadership of the late T. Wingate Todd in 1931 and continued by the Brush Foundation until the summer of 1942, the time originally planned for termination of the project. The children in the Series were selected for freedom from physical and mental defects, and on promise by their parents to continue participation in the project. They were somewhat above average in educational and economic status, were born in the United States, and almost all were of North European ancestry. The children were examined at three month intervals during the first year, at 6 month intervals up to 5 and annually thereafter. Series of 2 to 20 hand films from successive examinations of each of 1000 children were used for the Atlas.

The same Research Series was employed by Professor Todd for his Atlas of Skeletal Maturation of the Hand (1937) as far as possible but he was unable to obtain from it the stages during puberty and early adolescence. The authors of this Atlas had 6 more years of accumulation of films from the Series and were able to select all the standards from it.

The x-rays of the hands of individuals showing development typical for a particular age are reproduced as a series of standards, 28 for the male and 27 for the female, carrying them up to the age of 18. Although the stages usually represent regular intervals of

3 months, 6 months or a year, in some instances other intervals are introduced where the progress in development called for emphasis and did not correspond to the customary time.

As an indication of the reliability of the maturity shown in x-ray films of the hand and wrist, the authors illustrate the correspondence between such x-rays and sexual maturity in cases of precocious puberty and hypogenital states. They point out also that such x-rays reveal poorly mineralized skeletons, imbalance in skeletal development and scars of past illness.

In addition to the standards, that is, x-rays of the whole hand and wrist, there is a section which illustrates in outline drawings the maturity indicators for each individual bone. In the legend for the plate of each standard, the aberration of the skeletal age from the chronological age is pointed out for individual bones. Interpretation of the variations and interpolation for ages between the standards are materially helped by these illustrations of maturity indicators.

Although this volume will probably appeal particularly to clinical workers in fields of child development it will be very useful as a reference for the anatomist who may be called in consultation. It will also be valuable for the student in anatomy who wishes to observe the progress of ossification of the bones of the wrist and hand.

The book is rather sumptuously printed on expensive heavy paper, with pages measuring  $31 \times 23$  cm. The x-rays are technically good and carefully reproduced.

C. M. G.

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ANGEWANDTE UND TOPOGRAPHISCHE ANATOMIE. By GIAN TÖNDURY. 1949. 416 pages, 134 colored plates and 238 text figures. Fretz and Wasmuth, Zürich.

In this new Applied Anatomy by the Professor in Ordinary at the University of Zürich, the material has been selected for its practical value to medical students and practitioners of medicine, especially surgeons. The applications to clinical subjects, however, are given briefly and the anatomical point of view is consistently maintained.

The most striking feature of the book is its illustration by colored plates taken from drawings by Paul Winkler. According to the Foreword, the latter is a senior candidate in medicine who was able "to contribute his masterly anatomical and clinical understanding as well as his artistic talent." This is the first time that these drawings have been used and the dissections were prepared especially for this work.



The plates are of outstanding value for their technical excellence, accuracy and choice of subject matter. They range over the entire body. Recent trends in surgery show their influence in the timely emphasis on pictures of the lungs and autonomic nervous system. Most of the poses and details of the dissections are original and distinctive, and some give evidence of much skill and ingenuity in the arrangement of parts so that a large number of structures can be included in one drawing without undue distortion. A few antiques of rather wornout significance, such as the adductor canal through a window dissection, seem to have found their way in as an obeisance to tradition. On the other hand, the more practical subject of the femoral veins is given only in diagram. There is a scattering of appropriate cross and sagittal sections, and x-rays.

The text follows conventional lines both in choice of material and presentation. It is divided into three sections, the first, occupying about half the book, deals with the trunk; the second with the head and neck; the third with the extremities. The text is concise, reasonably complete and is illustrated with drawings and diagrams, borrowed for the most part, to illustrate various specific points. Attempts are made to emphasize the relationships of structures to each other but they consist primarily of lists of names. Two systems which are of especial interest to surgeons, the fasciae and the lymphatics, are rather sketchily and, in the case of the former, inaccurately portrayed. For example the scrotum is lined with cremasteric fascia and the fascia nuchae is superficial to the Trapezius.

The nomenclature is Latin following the N.K. revision of the B.N.A. which has not been widely accepted by American readers. Although this nomenclature may be scientifically correct and is therefore desirable theoretically, such terms as *vena cava caudalis* and *nervus thoracicus ventralis* will be difficult for people in this country to absorb.

There is an unbelievable confusion in the numbering of the figures. For example, figure 15 is a colored plate opposite page 24; 15-a is an x-ray on the back of this plate; figures 18, 19, 20 and 21 are text figures on pages 20, 29, 31 and 32 but figures 16 and 17 are colored plates of cross sections both on the page facing 32; figure 22 is an x-ray on the back of this plate. Four colored plates between pages 36 and 37 are, in order, figures 26, 24, 25 and 27. No excuse for this is apparent; it is disconcerting and detracts noticeably from the book because it persists throughout. Serial dissections of the hand on consecutive pages are numbered 319, 322, 324 and 326!

The reproduction of the colored plates is superb. The red, blue and yellow inks are bright but blend pleasingly. The paper is of

very good quality and the binding strong. This book is unhesitatingly recommended; the plates alone make it an excellent and surprisingly complete atlas of human anatomy.

C. M. G.

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AN AUTOBIOGRAPHY. By SIR ARTHUR KEITH. 1950. v + 721 pages with 6 plates, Price 25/-. C. A. Watts and Company, London.

Autobiographies of Anatomists are rare, and good ones are still rarer. For the life of a scientist is usually not very glamorous, as most of us know only too well. Hence only few scientists reach positions which make their life interesting for the average reader. Sir Arthur Keith belongs to this small group. Born and raised on a Scottish farm, then settling in London when the British Empire was at the height of its glory, and taking part in many of the burning issues of the day, Keith has led a full life as one of the spiritual leaders of a great nation. But the good common sense of the Scotch farmer, used to looking things squarely in the face, remained with Arthur Keith throughout his life.

Arthur Keith was born in 1866, the 6th child (three brothers and two sisters had preceeded him) to Scottish farmers, raised during his first 8 years on Quarry Farm near Aberdeen on the bank of the Don, and then on Kinnermit Farm near Turriff, in the valley of the Deveron. Arthur's life seems to have differed in no way from that of many other Scotch lads, and he tells us that for the greater part of 1882 (when he was 16) he was at home, "taking his place among the men in the fields, and had set his heart on becoming a farmer." But at the end of that year, a young stranger, John Kemp, knocked at the door of the Keiths and asked for lodgings, explaining that he had been engaged to coach the young laird for his exams. Mrs. Keith gave him the spare room, and it was only natural that Kemp would see friends of his, and that young Arthur would come to know this group. He envied these young students, "not because they could lie abed to any hour, . . . nor because they fared well but because of the wonderful life they had as students in the University." It was only after Arthur had decided on the University for himself that he determined to take the medical road to this most desirable paradise. He saw it through, graduated from Marischal College in 1888 and set out to seek a livelihood by the practice of Medicine. His first post was with a Dr. Urquhart, in a fashionable asylum near Perth. It did not last long. To give, in the

absence of his chief, the official cause of death as "suicide" was more than the fastidious Dr. Urquhart could stand. The reputation of his institution, so one gathers, was at stake, and a polite letter to Dr. Keith, relieving him of his duties because of his (Keith's) ill health enabled the director to look for a more understanding assistant. Arthur Keith went back to the farm where he helped to keep things in order, and looked around for a new opening. Finally he found one, across the border in England. But that did not last too long either, although for different reasons. A letter from his old professor, with an enclosure by Sir Joseph Hooker arrived offering him a well-paid post with the "Gold Fields of Siam." Keith was chosen by Professor Trail because Hooker wanted the young doctor to have botanical leanings and to collect specimens. Siam presented one great problem to the young doctor: malaria. It was clearly endemic among the natives. Did the monkeys have it, too? Keith set out into the jungle and began to hunt for monkeys. Let Sir Arthur take over: "As I dissected this specimen all my old love for Anatomy was roused; what a waste of rare anatomical material were I to restrict my attention to spleen and blood!" Keith became a subscriber to the *Journal of Anatomy and Physiology*, and worked hard in his little laboratory in the jungle. In September 1889 he enters in his diary his plans for the future: at the end of three years when he will have enough money laid aside he will study for two more years and get his M.D. and F.R.C.S. "My goal was Anatomy; I was to make a living by teaching medical students the structure of mankind and devote my research to the elucidation of the origin of his body." At that time Arthur Keith was 23 years old. He subsequently became a teacher at London Hospital, Conservator of the Museum of the Royal College of Surgeons, now lives in retirement at Down House in Kent. The book opens with a charming account of the daily life of Sir Arthur at Down House, how he works (in spite of his eighty-odd years) on the farm, spends several hours at his desk and enjoys the company of friends at other times.

What kind of man is the author? Several traits come out so clearly when one reads the narrative that the question is perhaps not as foolish as it might sound at first. It is said that the Scotch are the only people on earth who can take a joke on themselves. That Keith can is obvious from many instances. Thus when he talks about the Piltdown skull he ends up by stating: "the irony of the situation lay in this—the evidence which the future was to bring proved so strong that I had to accept their (his scientific opponents) conviction." Behind this, there is an outstanding sincerity. Keith



seems singularly free of favourable prejudices about himself. "I never became an efficient lecturer . . . my voice which has no penetrating quality was apt to drop at the end of a sentence, leaving my listeners guessing as to what I had said." As one who listened to many lectures of Arthur Keith during the years to which he alludes in the paragraph just quoted, I can vouch for its incorrectness. Keith spoke in a conversational tone, but slowly and clearly, and every word he uttered had a meaning and reflected his lucid thoughts so that at the end of the lecture, one had a clear grasp of the argument and remembered it. Keith is perhaps the kindest man one can think of. It is a little difficult to quote chapter and verse for this statement, but the attentive reader will be struck by it on page after page. "The brain is much more than an organ for passing examinations: it is the organ of feeling and of doing—functions which are difficult to measure." It is precisely this subtle sense of feeling and doing that make the charm of this autobiography.

To review Keith's achievements in this note would be impossible, and would be carrying coals to Newcastle in any case. His wide interests, his many activities as a lecturer to learned bodies and otherwise, particularly when he had reached "that degree of standing which brought invitations to give opening addresses and to distribute prizes in the medical schools of London," and with it the wide circle of friends which gathered around the Keiths, make good reading even for those who are not anatomists.

The reviewer still remembers Keith at the Museum of the R.C.S. On home during the vacations, I made daily pilgrimages to Lincoln's Inn Fields to study the beautiful collection of brains in the Museum. Jar after jar was taken off the shelves and put on a little board given me by a kind attendant. Every now and then I would see an elderly gentleman (so he seemed to me then, his portrait on p. 312 shows how we judge age from the variable reference point of our own), with a remarkably kind expression, go through the galleries. One day he stopped by me, and invited me in the course of the short conversation to come up to one of the work rooms where I could pursue my studies in greater comfort. This was the beginning of a friendship that has lasted, I think I can rightly say, through all these years. It was Keith who saw to it that I could embark on an anatomical career.

As one looks with Keith over his life, two things stand out: to be an anatomist one must have heard the call, and to be a good anatomist one should have gone through at least some years of medical work. How else can one guide the first steps of medical students?

GERHARDT VON BONIN



## BOOKS RECEIVED

- A Textbook of Neuro-anatomy* by Albert Kuntz. 5th edition, 1950. 524 pages, 331 figures, \$8.00. Lea and Febiger, Philadelphia.
- Comparative Anatomy Laboratory Manual* by Lloyd Raymond Gribble. 1950. xii + 231 pages, illustrated, \$3.00. The Blakiston Company, Philadelphia.
- Embryology* by Lester George Barth. 1949. xii + 330 pages, 194 figures, \$5.00. The Dryden Press, New York.
- An Atlas of Cat Anatomy* by Hazel E. Field and Mary E. Taylor. 1950. 75 pages, 57 plates, \$3.75. The University of Chicago Press, Chicago.
- Radiographic Atlas of Skeletal Development of the Hand and Wrist* by William Walter Greulich and S. Idell Pyle. 1950. xiii + 190 pages, illustrated, \$10.00. Stanford University Press, Stanford, California.
- Histology* by Arthur Worth Ham. 1950. xix + 756 pages, 445 figures, \$10.00. J. B. Lippincott Company, Philadelphia.
- Biological Actions of Sex Hormones* by Harold Burrows. 2nd edition, 1949. xiii + 615 pages, \$8.50. Cambridge University Press, London and New York.
- Selected Invertebrate Types*, edited by Frank A. Brown, Jr. 1950. xx + 597 pages, 235 figures, \$6.00. John Wiley and Sons, New York.
- Introduction to Neuropathology* by Samuel Pendleton Hicks and Shields Warren. 1950. xiii + 494 pages, illustrated, \$10.00. McGraw-Hill Book Company, New York.
- Die Sektion des Gehirns und Rückenmarks und ihrer Hüllen* by Berthold Ostertag. 2nd edition. 1949. iv + 47 pages, illustrated, DMark 6.60. Springer-Verlag, Berlin.
- Angewandte und Topographische Anatomie* by Gian Töndury. 1949. 416 pages, 134 colored plates drawn by Paul Winkler and 238 text figures. Fretz and Wasmuth Verlag, Zürich.
- Races . . . A Study of the Problems of Race Formation in Man* by Carleton S. Coon, Stanley M. Garn and Joseph B. Birdsell. 1950. xiv + 153 pages, illustrated, \$3.00. Charles C. Thomas, Publisher, Springfield, Illinois.
- Problems of Morphogenesis in Ciliates. The Kinetosomes in Development, Reproduction and Evolution* by André Lwoff. 1950. ix + 103 pages, illustrated, \$2.50. John Wiley and Sons, New York.
- The Cerebral Circulation in Health and Disease* by Carl F. Schmidt. 1950. v + 78 pages, \$2.00. Charles C. Thomas, Springfield, Illinois.
- A Manual of Practical Vertebrate Morphology* by J. T. Saunders and S. M. Manton. 2nd edition, 1949. viii + 255 pages, illustrated, \$4.75. Oxford University Press, London.
- Plant Viruses* by Kenneth M. Smith. 2nd edition, 1948. ix + 78 pages, illustrated, \$1.50. Methuen's Monographs on Biological Subjects, Methuen & Co., London; John Wiley & Sons, New York.

- Mendelism and Evolution* by E. B. Ford. 5th edition, 1949. xii + 122 pages, \$1.25. Methuen's Monographs on Biological Subjects, Methuen & Co., London; John Wiley & Sons, New York.
- The Classification of Animals* by W. T. Calman. 1949. vii + 54 pages, \$1.25. Methuen's Monographs on Biological Subjects, Methuen & Co., London; John Wiley & Sons, New York.
- Atlas of Human Anatomy, Descriptive and Regional* by M. W. Woerdeman. 1950. Volume 2, pages not numbered, 642 figures + index. \$10.00; Volumes 1 and 2, set, \$18.00. The Blakiston Company, Philadelphia.
- Buchanan's Manual of Anatomy*, edited by F. Wood Jones, 8th edition, 1950. viii + 1616 pages, 847 figures. \$8.50. The Williams and Wilkins Company, Baltimore.
- Essay on the Cerebral Cortex* by Gerhardt von Bonin. 1950. xiii + 150 pages. \$3.75. Charles C. Thomas, Springfield, Illinois.
- Enzymes, Growth and Cancer* by Van R. Potter. v + 64 pages. \$1.85. Charles C. Thomas, Springfield.
- Grundriss der Histologie und mikroskopischen Anatomie des Menschen* by Hans Petersen, 4th edition by Fritz Körner, 1950. vii + 178 pages. DM 8.40. Springer-Verlag, Berlin.
- A Textbook of Histology, Functional Significance of Cells and Intracellular Substances* by E. V. Cowdry. 4th edition, 1950. 640 pages, illustrated, \$8.00. Lea and Febiger, Philadelphia.
- Asphyxia Neonatorum, its Relation to the Fetal Blood, Circulation and Respiration and its Effects upon the Brain* by William F. Windle. 1950. vii + 70 pages, 9 figures, \$2.00. Charles C. Thomas, Publisher, Springfield, Illinois.
- The Philosophy of Edmund Montgomery* by Morris T. Keeton. 1950. xi + 386 pages. Southern Methodist University Press, Dallas, Texas.
- The Chordates* by Herbert W. Rand. 1950. xi + 862 pages, 609 figures, \$6.00. The Blakiston Company, Philadelphia.
- McClung's Handbook of Microscopical Technique, for Workers in Animal and Plant Tissues*, edited by Ruth McClung Jones, 3rd edition, 1950. xix + 790 pages, 142 figures, \$12.00. Paul B. Hoeber, New York.
- The Esophagus and Pharynx in Action, a Study of Structure in Relation to Function* by William Lerche. 1950. xii + 222 pages, 92 figures, \$5.50. Charles C. Thomas, Publisher, Springfield, Illinois.
- On the Experimental Morphology of the Adrenal Cortex* by Hans Selye and Helen Stone. 1950. viii + 105 pages, illustrated. \$2.25. Charles C. Thomas, Publisher, Springfield, Illinois.
- Selected Studies on Arteriosclerosis* by Rudolf Altschul. 1950. xii + 182 pages, 79 figures. \$5.50. Charles C. Thomas, Publisher, Springfield, Illinois.
- Physiology and Anatomy* by Esther M. Greisheimer. 1950. xvi + 841 pages, 478 figures. \$4.00. J. B. Lippincott Company, Philadelphia.
- Evaluation of Industrial Disability* by Packard Thurber, editor. 1950. vi + 89 pages, 8 figures. \$4.00. Oxford University Press, New York.